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ROLE OF GLP-1 IN ENERGY HOMEOSTASIS

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presented by

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«Poor old Pyecraft! Great, uneasy jelly of substance! The fattest clubman in London.

"I expect," he said, "you take no more exercise than I do, and probably you eat no less." (Like all excessively obese people he fancied he ate nothing.) "Yet,"–and he smiled an oblique smile– "we differ."

And then he began to talk about his fatness and his fatness; all he did for his fatness and all he was going to do for his fatness; what people had advised him to do for his fatness and what he had heard of people doing for fatness similar to his. "A priori," he said, "one would think a question of nutrition could be answered by dietary and a question of assimilation by drugs." It was stifling. It was dumpling talk. It made me feel swelled to hear him.

"I'd give anything to get it down," he would say–"anything," and peer at me over his vast cheeks and pant.

[...]

"So far as I–can make it out, this is a recipe for Loss of Weight. ("Ah!" said Pyecraft.)
"Let me try it," said Pyecraft.
"It's nasty stuff," I said.
"No matter," he said, and took it.»

Herbert George Wells

The Truth About Pyecraft
(From Twelve Stories, 1903)

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CONTENTS

LIST OF ABBREVIATIONS

α-MSH	alpha melanocyte stimulating hormone
AgRP	agouti-related peptide
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variances
AP	area postrema
ARC	arcuate nucleus of the hypothalamus
AUC	area under the curve
BAT	brown adipose tissue
BBB	blood brain barrier
BDNF	brain derived neurotropic factor
BMI	body mass index
BTS	biotinylated tyramine solution
BW	body weight
CAA	central autonomic area
CART	cocaine- and amphetamine-regulated transcript
CBS	caudal brainstem
CCK	cholecystokinin
CD	standard chow diet
cDNA	complementary deoxyribonucleic acid
CNO	clozapine-N-oxide
CNS	central nervous system
CVO	circumventricular organ
DIO	diet-induced obesity
DMEM	Dulbecco's modified Eagle Medium
DMH	dorsomedial hypothalamus
DMSO	dimethyl sulfoxide
DMV	dorsal motor nucleus of the vagus nerve
DPP-IV	dipeptidyl peptidase-IV

DREADD	designer receptor exclusively activated by designer drugs
Ex-4	exendin-4
Ex-9	exendin-(9-39)
FACS	Fluorescence activated cell sorting
FDA	Federd Drug Adminstration
FI	food intake
Fig	figure
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GI	gastrointestinal
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
GLP-1*R*	glucagon-like peptide-1 receptor
GLP-2	glucagon-like peptide-2
Glu-Cre	glucagon promoter - Cre recombinase
Glu-YFP	glucagon promoter - yellow fluorescent protein
HEK-293	Human embryonic kidney cells 293
HFD	high-fat diet
HPV	hepatic portal vein
ICV	intracerebroventricular
IL-6	interleukin-6
IML	intermedialateral cell column
IP	intraperitoneal
*JAK*2	Janus-activated kinase
KD	knockdown
LepR	leptin receptor
LFD	low fat diet
LHA	lateral hypothalamic area
LRA	leptin receptor antagonist
LV	lentivirus
*MC*4*R*	melanocotrin-4 receptor
MLD	mesenteric lymph duct

NAc	nucleus accumbens
NPY	neuropeptide-Y
*NTRK*2	neurotrophic tyrosine kinase 2
NTS	nucleus of the tractus solitarii
OECD	organizatuin fir economic co-operation and development
*P-STAT*3	phosphorylated STAT3
PBS	phospate buffered saline
PBST	0.3% Triton-X 100 in PBS
*PC*1/3	protein convertase 1/3
*PC*2	protein convertase 2
PCR	polymerase chain reaction
*PCSK*1	proconvertase 1
PEG	polyethylene glycol
PFA	paraformaldehyde
*Phox*2*b*	paired-like homeobox 2b
PKA	protein kinase A
PMCH	pro-melanin concentrating hormone
POMC	proopiomelanocortin
PPG	preproglucagon
PVN	paraventricular nucleus of the hypothalamus
PYY	peptide tyrosine tyrosine
qPCR	quantitative polymerase chain reaction
RER	respiratory exchange ratio
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcript – polymerase chain reaction
SDA	subdiaphragmatic vagal deafferentation
SEM	standard error of the mean
shRNA	short hairpin RNA
*SIM*1	single-minde homolog 1
*SOCS*3	suppressor of cytokine signaling 3
*STAT*3	signal transducer and activator of transcription 3

VMH	ventromedial nucleus of the hypothalamus
$VO2$	rate of oxygen consumption
VTA	ventral tegmental area
W	week(s)
WAT	white adipose tissue
WHO	world health organization
WT	wild type
YFP	yellow fluorescent protein

SUMMARY

Obesity has become a great problem worldwide. According to world health organization reports in 2016, more than 1.9 billion adults were overweight worldwide and over 650 million of these were obese. Obesity has both instant (psychological) and long-term health consequences. It is linked to various diseases including cardiovascular diseases, type-2-diabetes, etc. Therefore, growing public health concerns emphasize the need to develop a strategy to treat or – better - prevent obesity.

Obesity develops if energy intake is greater than energy expenditure over a longer time-period. A better understating of the gut-brain interactions in the context of energy homeostasis with the focus on food intake (FI) may help to find novel solutions to treat and prevent obesity. One of the intestinal peptides that has the capacity to control energy intake and metabolism is glucagon-like peptide-1 (GLP-1), which is produced in the periphery by enteroendocrine L-cells and centrally in pre-pro glucagon (PPG) neurons.

One of our goals was to gain some basic knowledge about the role of centrally produced GLP-1 in energy homeostasis (Chapter 2). To this end, we generated a lentivirus (LV)-mediated PPG knockdown (KD) mouse model and characterized its phenotype. The KD resulted in decreased body weight gain and daily FI in HFD-fed mice compared to their corresponding virus-injected controls. We found a decrease in hypothalamic NPY mRNA expression in KD compared to control animals. These findings suggest that PPG neurons can stimulate FI through NPY signaling.

In addition, we addressed the question of PPG neuronal activation and silencing using the 'designer receptors exclusively activated by designer drugs' (DREADD) technology. Acute DREADD-mediated activation of PPG neurons decreased FI while silencing had the opposite effect. We also addressed the effects of PPG neuronal activation on energy expenditure and observed that it results in a decrease of respiratory rate and an increase in heat loss. Together, these findings indicate that PPG neurons play an important role in energy homeostasis and suggest that their function is more complex than commonly thought.

The experiments described In Chapter 3 focused on peripheral GLP-1. We used an unrestrained rat model to collect intestinal lymph and hepatic portal vein (HPV) blood with the aim of precisely measuring the release of

endogenous intestinal GLP-1 in relation to isocaloric meals with a different fat content. In addition, we aimed at addressing the possible physiological relevance of IP administered GLP-1.

With the aim to evaluating whether intestinal lymph would provide a better readout than HPV plasma for the secretion of intestinal peptides and metabolites, we measured in both compartments, i.e., in the mesenteric lymph duct (MLD) and in the HPV, in parallel at different time points the concentrations of GLP-1, insulin, glucose, and triglycerides (TG) associated with different test meals and exogenously administered GLP-1. In addition, we characterized the dipeptidyl peptidase-IV (DPP-IV) activity to evaluate its possible influence on the GLP-1 concentrations measured in MLD lymph and HPV plasma. DPP-IV activity in HPV plasma was higher than in intestinal lymph, which presumably contributed to the lower levels of GLP-1 in HPV plasma compared to MLD lymph. Insulin and glucose showed similar profiles in MLD lymph and HPV plasma, whereas TG levels were of course higher in lymph than in plasma. Our findings indicate that intestinal lymph provides a sensitive readout for intestinal GLP-1 release when fat-rich diets are consumed. While it is less interesting to monitor insulin and other metabolites in intestinal lymph, it may still be useful for some actions of gut peptides under certain conditions.

The General Discussion Chapter 4 extends the interpretations of our own findings, discusses their limitations and puts them into a broader perspective. It also identifies new questions that our findings raise, and discusses future directions that research in this area might take. Our findings and animal models add to the basic knowledge about the role of GLP-1 and contribute to mounting evidence that central GLP-1 producing PPG neurons may eventually become a promising therapeutic target for the fight against obesity.

ZUSAMMENFASSUNG

Übergewicht ist zu einem weltweiten Problem geworden. Den Berichten der Weltgesundheitsorganisation von 2016 zufolge, sind mehr als 1.9 Milliarden Erwachsene übergewichtig und von diesen über 650 Millionen adipös. Übergewicht hat sowohl unmittelbare psychische als auch langfristige (psychische und physische) Gesundheitsfolgen. Es steht im direkten Zusammenhang mit verschiedenen Krankheiten wie Herz-Kreislauf-Erkrankungen, Typ-2-Diabetes usw. Im Interesse der öffentlichen Gesundheit ist es deshalb notwendig, eine wirksame Strategie zur Behandlung oder - besser - Vorbeugung von Adipositas zu entwickeln.

Übergewicht entsteht, wenn über längere Zeit die Energieaufnahme grösser ist als die -abgabe. Ein besseres Verständnis der Interaktionen zwischen Darm und Gehirn in Bezug auf die Energiehomöostase und inbesondere mit dem Fokus auf die Nahrungsaufnahme könnte helfen, neuartige Lösungen zu finden, um Übergewicht zu behandeln und vorzubeugen. Eines der Intestinalpeptide, welches die Möglichkeit bietet, die Energieaufnahme und den Metabolismus zu kontrollieren, ist Glucagon-like peptide-1 (GLP-1). GLP-1 wird peripher von enteroendokrinen L-Zellen und zentral von Pre-proglucagon (PPG) Neuronen produziert.

Eines unserer Ziele war es, das Grundlagenwissen über die Rolle von zentral produziertem GLP-1 bei der Regulation der Energiehomöostase zu vermehren (Kapitel 2). Dazu generierten wir mittels Lentiviren ein Mäusemodel mit einem PPG Knockdown (KD) und charakterisierten dessen Phänotyp. Verglichen mit den virusinjizierten Kontrollen führte der Gen-Knockdown bei Mäusen, die mit einer Hochfettdiät gefüttert wurden, zu einer verminderten Körpergewichtszunahme und zu einer reduzierten Nahrungsaufnahme. Zudem stellten wir bei den KD-Mäusen im Vergleich zu den Kontrollen eine verminderte mRNA Expression von NPY im Hypothalamus fest. Diese Befunde deuten darauf hin, dass PPG Neurone die Nahrungsaufnahme über NPY Signalwege stimulieren.

Darüber hinaus untersuchten wir die Frage der Aktivierung und Deaktivierung von PPG Neuronen mittels der 'designer receptors exclusively activated by designer drugs' (DREADD) Technologie. Die akute Aktivierung der PPG Neurone mittels DREADD führte zu einer reduzierten Nahrungsaufnahme, während die Deaktivierung den gegensätzlichen Effekt hatte.

Wir erfassten auch die Energie während der Aktivierung von PPG Neuronen und stellten eine Reduktion der Atemfrequenz und einen erhöhten Wärmeverlust fest. Diese Resultate weisen darauf hin, dass PPG Neurone eine wichtige Rolle bei der Energiehomöostase spielen und lassen vermuten, dass deren Funktion komplexer ist als bisher angenommen.

Die in Kapitel 3 beschriebenen Experimente konzentrierten sich auf peripheres GLP-1. Wir verwendeten hierfür ein Modell, bei welchem sich die Ratten während dem Sammeln von intestinaler Lymphe und Pfortader (HPV) Blut uneingeschränkt bewegen konnten. Das Ziel war die exakte Messung der Ausschüttung von endogenem intestinalem GLP-1 während isokalorischen Mahlzeiten mit verschiedenem Fettgehalt.

Ein weiteres Ziel war, zu evaluieren, ob die intestinale Lymphe ein besseres Readout für die Sekretion von intestinalen Peptiden und Metaboliten ist als HPV Plasma. Wir erfassten dazu parallel und zu verschiedenen Zeitpunkten, die Konzentrationen von GLP-1, Insulin, Glukose und Triglyceriden (TG) in Relation zu verschiedenen Testmahlzeiten und nach exogen verabreichtem GLP-1 im Pfortaderblut und in Lymphe aus dem mesenterialen Lymphkanal.

Zusätzlich charakterisierten wir die Aktivität der Dipeptidylpeptidase -4 (DPP-IV), um eine mögliche Beeinflussung der GLP-1 Konzentrationen in der Intestinallymphe und im HPV Plasma zu evaluieren. Die DPP-IV Aktivität im HPV Plasma war höher als in der Intestinallymphe, was wahrscheinlich zu den geringeren GLP-1 Konzentrationen im HPV Plasma im Vergleich zu den Konzentrationen in der Intestinallymphe beitrug. Insulin und Glukose wiesen in der Intestinallymphe und im HPV Plasma ein ähnliches Profil auf, während dieTG, wie erwartet, in der Lymphe höher waren als im Plasma.

Unsere Resultate weisen darauf hin, dass Intestinallymphe ein sensitiver Readout für die intestinale GLP-1 Freisetzung ist, wenn fettreiche Nahrung konsumiert wird. Weniger interessant ist es, Insulin und Metabolite in der Intestinallymphe zu messen. Die Messung von Fettmetaboliten könnte unter bestimmten Bedingungen und für spezielle Fragestellungen (Fettabsorption, etc.) jedoch nützlich sein.

Die allgemeine Diskussion in Kapitel 4 erweitert die Interpretationen unserer eigenen Befunde, diskutiert deren Beschränkungen und stellt sie in einen grösseren Rahmen. Zudem werden auch neue Fragen, die unsere Befunde aufwerfen und zukünftige Richtungen diskutiert, in welche die Forschung in diesem Bereich gehen könnte. Unsere Befunde vermehren den Kenntnisstand bezüglich der Rolle von GLP-1 bei der Regulation

der Energiehomöostase und unterstützen sich mehrende Hinweise darauf, dass zentrale, GLP-1 produzierende Neurone ein valables Ziel für die Therapie der Adipositas sein könnten.

1

GENERAL INTRODUCTION

1.1 OBESITY AS WORLDWIDE PROBLEM: TENDENCIES, POSSIBLE CAUSES AND VIABLE APPROACHES

The media and the scientific community refer to obesity as an epidemic. Obesity prevalence increased dramatically over the past decades, and such a fast change can not be simply attributed to genetics. Our modern life style, which involves only little exercise and an abundance of food with high energy density and often poor nutritional value are the main factors to blame [90, 97, 98, 105, 160]. Even though the most obvious and least invasive solution would be to incorporate more exercise in our daily life and to change our diet, human nature tends to resist such changes, and the majority of us still dream about other, simpler solutions. Adhering to a diet for a lifetime is probably one of the most difficult rules to keep. Hence, we are waiting for a 'magic pill' that would solve all our problems, allowing us to eat as much as we want and, should we not like our picture in the mirror or get health complications due to the overweight, have a drug to solve these problems for us as well. If this medicine would be of natural, organic origin, it might conquer consumers of all believes. But, as much as we wish to have such a solution, as far we are still away from achieving it.

To have the fundamental scientific knowledge about what causes obesity and what changes in the body once we reach the obese status, is the key to finding a viable approach to combatting obesity and its metabolic consequences. My work in the lab related to gaining further knowledge in the field of obesity, with the focus on the role of glucagon-like peptide-1 (GLP-1) in the control of food intake (FI) and regulation of energy homeostasis.

1.1.1 *Short overview of current on obesity trends worldwide*

Obesity is an excessive accumulation of body fat. It is linked to several health problems, such as type-2-diabetes mellitus (T2DM), metabolic syndrome, cardiovascular diseases, and some types of cancer [100]. Judged

by the so called BMI (calculated by the formula $weight(kg)/height^2(m^2)$), which aims at quantitatively assessing the amount of tissue mass relative to an individual's height, people can be categorized as underweight (< 18.5), normal weight (18.5-24.9), overweight (> 25), or obese (> 30) [20]. The BMI does, however, not asses the tissue or body composition, i.e., the ratios among the amount of muscles, fat and bones. Knowing a person's weight and height is therefore not sufficient to judge an individual's true obesity status. For example, an athlete with lots of muscles falls into the same BMI category as an obese person with the same height and weight, but much less muscle mass [56, 89]. Nevertheless, despite its obvious limitations, the BMI is still the most widely used method to judge on the overall obesity status of the population. And globally the figures are daunting. Obesity has increased by approximately 40% between 1980 and 2013 [109]. According to the World Health Organization (WHO) worldwide `http://www.who.int/gho/ncd/`:

- 39% women and 39% of men were overweight at the age of 18+ in 2016
- 18% of children and adolescents were overweight or obese in the age range of 5-19 in 2016

Childhood obesity remains one of the biggest challenges to be tackled, otherwise the number of obese individuals will continue to increase over time, and the burden for our health care systems will be daunting [6, 67, 85, 93].

The level of adiposity increases in all countries, making it a truly global problem [18, 41, 119, 143]. Women and men, both, are similarly susceptible to develop obesity, i.e., obesity is not a sex or gender-specific problem. It is therefore reasonable to consider women and men together and look at the statistics of individual countries over time. The obesity trends over time reveal that the USA together with Mexico are taking the lead, followed by Australia and England Fig. 1.1.

Unfortunately, the awareness of the obesity pandemic does not do anything to alleviate it. This failure to deal with a recognized problem by behavioural modification calls for a better understanding of the causes that lead to such a profound increase in obesity worldwide. It is necessary to direct the efforts of research towards possible solutions to slow down these trends and to eventually be able to prevent it and perhaps also help those

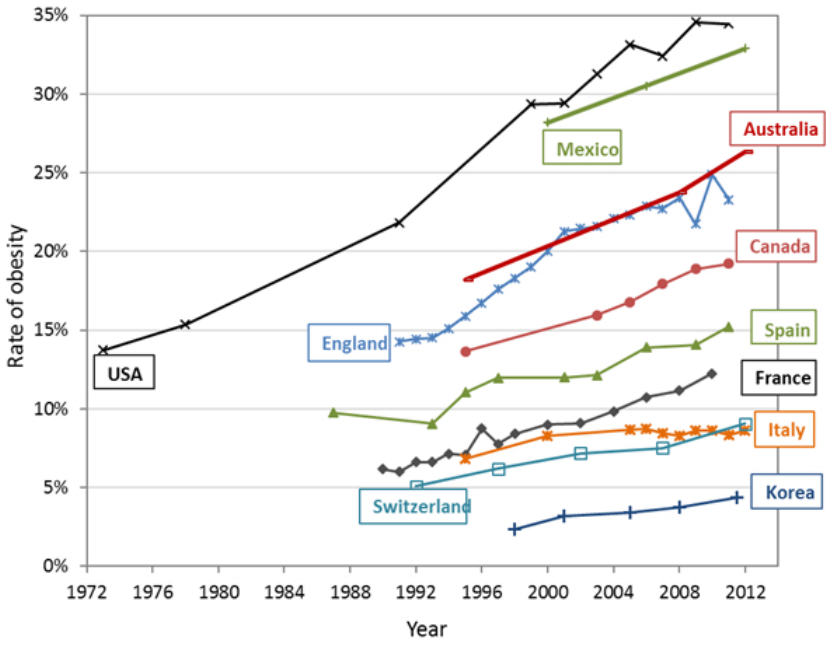

FIGURE 1.1: Obesity rates in different countries reported as rate of obesity in percentage versus time in years. Organization for Economic Co-Operation and Development (OECD) analysis of health survey data 2014. Note: Measured height and weight in Australia, England, Korea, Mexico and the United States; self-reported in other countries. Source: `http://www.oecd.org/newsroom/more-efforts-needed-to-tackle-rising-obesity.htm`

people who already are obese and are facing the health complications associated with it.

1.1.2 *Limitations of epidemiological data*

It is worth mentioning that epidemiological data are limited in several ways. First, in most cases weight and height data for the BMI score are self-reported rather than measured across the population. This can lead to bias (as mentioned in relation to the OECD survey data) because women are less willing to report their true BW compared to men. Second, not all people categorized as obese based on BMI are actually unhealthy [53]. Rather, a person can have a substantial accumulation of fat and yet be considered healthy in regards to metabolic as well as cardio-respiratory fitness ('obesity paradox'), and in some cases even carry a lower risk of all-cause mortalities than people with normal BMI [13, 53, 113, 157]. The biggest limitation of epidemiological studies is that they can not take into consideration all complex influencing factors [66].

1.1.3 *Genetics*

It is widely accepted that one of the main contributors to developing obesity is our modern life style, but it is also clear that a genetic component determines the risk to develop an obese phenotype under these conditions [58]. Studies in monozygotic twins revealed high heritability values for the BMI, even if the twins grew up in different environments [165]. Studies in adopted children revealed that the children's BMI correlates better with their biological parents' BMI than with their adopted parents' BMI [138, 141].

Monogenetic forms of obesity are rare and may play a role in up to 5% of obese individuals [3,52]. Over 200 single gene mutations in homozygous form can be associated to human obesity (all these mutations are found in only 10 genes), for example leptin, leptin receptor, pro-opiomelanocortin (POMC), proconvertase 1 (PCSK1), melanocortin-4 receptor (MC4R), brain-derived neurotropic factor (BDNF), neurotrophic tyrosine kinase 2 (NTRK2), and single-minded homolog 1 (SIM1) [3,12,104,123]. Interestingly all these genes code for proteins involved in the leptin-melanocortin signaling pathway in the hypothalamus, which emphasizes the importance of this pathway for energy homeostasis [3,43,52].

Polygenetic forms of obesity are, however, much more frequent; usually the combination of several altered genes, which can vary greatly among individuals, accounts for an enhanced susceptibility to gain weight in our current obesogenic environment. The underlying causes are therefore complex and require further attention [43, 165].

1.1.4 *Evolutionary predisposition towards obesity*

The so-called 'thrifty gene'- theory proposes that the capability to store large amounts of fat could have been an advantage for early humans during the hunting and gathering time [107, 108]. In modern society, however, this trait is disadvantageous, because food is constantly available and, hence, the propensity to store large amounts of fat might lead to obesity [140].

The 'thermogenesis hypothesis' states that climate changes influenced the genes. In fact, around the world differences with respect to the prevalence of obesity between warm and cold-adapted ethnic groups can be seen. Thus, people living in warm climates have a higher obesity prevalence than those living in cold climates. Body shape, overall body surface and thermogenesis could be related to climate [135].

Although all these hypotheses have some valid arguments, none can explain the wide range of obesity forms. Nevertheless, it is still worth to consider human evolution for a better understanding of modern human eating behavior leading to obesity. For example, humans have not developed alone. Rather, they have co-existed with certain microorganisms for millions of years. Hence, it is also interesting to consider the modifications that these microorganisms undergo nowadays and how our lifestyle changes affect them and how factors they release in turn affect us.

1.1.5 *Bacterial 'domination'*

Many different microorganisms live on the surface of the mucosa in the human intestine, i.e., they colonize our gut. The collection of all these microbes is called human gut microbiota [58, 120]. The gut microbiota supposedly represent more than 3 million non-redundant genes [120], which outnumbers the human genome by far $(x150)$ [120]. It should therefore not be surprising that microbiota and their changes contribute to various aspects of energy homeostasis, possibly also including the development of metabolic diseases such as obesity and T2DM [147, 163, 164]. In gen-

eral microbiota can influence many aspects of human metabolism, such as biosynthesis of the steroid hormones, bile salt metabolism, etc. [60,142,155, 158,168].

Analysis of human feces revealed that they contain mainly seven bacterial phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria [86, 147]. Changes in the ratio of these bacterial populations supposedly can affect energy homeostasis [29,71,158,164].

1.1.6 *Diet and lifestyle interactions in obesity*

Environmental factors such as life style and diet preferences influence body weight (BW). Sugar-sweetened beverage consumption has been implicated in the obesity epidemic, as has a high intake of fried foods, usually resulting in higher energy intake [20,66]. Additional factors are a high intake of saturated fatty acids, disturbed sleep, social factors, and a reduction in physical activity, which all combine to shape an individual's BW [20,105,119].

1.2 APPROACHES TO COMBAT OBESITY

1.2.1 *Use of weight-reducing drugs*

For individuals with a BMI above 30 kg/m^2 drug treatments are considered as an option, especially if the high BMI is accompanied by complications such as T2DM, dyslipidemia or hypertension [100]. Different classes of anti-obesity drugs are currently approved by the US Food and Drug Administration (FDA). One class of drugs is designed to reduce absorption of calories. Orlistat (Xenical®, Roche) – inhibits the intestinal lipase and, hence, the intestinal absorption of triglycerides; other drugs act on FI through targeting appetite controlling pathways in the brain. For example, Lorcaserin HCl (Belviq®, Arena Pharmaceutics) – serotonin receptor agonist; Pramlintide (Symlin®, AstraZeneca) - amylin analog and Liraglutide (Saxenda®, Novo Nordisk) – GLP-1 analog; [28, 112]. Despite large number of drugs on the market, it is noteworthy that the maximum weight loss that can be expected to result from such medications is only in the range of 5-10 kg over 2 years [38], which doesn't seem much if somebody is heavily obese. One should also mention possible side effects of these drugs and the simple fact that they are not always effective. For example,

it is not clear which sub-population of patients will respond effectively to one or the other drug and at which doses. Hence, there is a need for more personalized pharmacologic intervention. Ideally, the pharmacological response should be assessed in relation to genetic tests. The recognition of this need gave rise to the field of pharmacogenetics [22]. Overall, however, at least until now, bariatric surgery rather than pharmacologic approaches, alone or in combination with diets, still is the most effective means against obesity and its metabolic consequences [136].

1.2.2 *Bariatric surgery*

Bariatric Surgery was first used over 60 years ago. Currently every year more than 200.000 bypass surgeries are performed alone in the USA [80, 110]. Originally, all the health benefits of bariatric surgery were attributed to a physical limitation of FI due to the reduction in stomach size together with malabsorption of nutrients [137, 144]. Interestingly, however, improvements in T2DM occur prior to the significant BW loss, which suggests that other factors such as gut hormones, bile acids, gut microbiota etc. may be involved [96, 134].

The bariatric surgery approach, however, has also shortcomings. Thus, several studies report that the capacity to absorb vitamin D and other nutrients is substantially reduced after surgery [35, 44, 48]. This and the fact that bariatric surgery is invasive, leads to gut remodeling and can entail infections, stomach obstruction or a constant feeling of nausea with frequent vomiting, calls for better methods to treat obesity [110, 129, 134]. Nevertheless, the undeniable metabolic benefits and the current unavailability of other, more efficient methods, account for the fact that bariatric surgery remains the most efficient and widely used method to combat obesity worldwide.

1.3 UNDERSTANDING THE CENTRAL AND PERIPHERAL CONTROL OF FOOD INTAKE

As mentioned before, what we eat and how much we eat can lead to obesity [12, 29, 66, 67, 75, 113, 138, 157]. It is therefore important to ask what makes us eat one or the other kind of food, how much and how often. Of course countless factors can influence eating, and as a result, our daily choices of food usually changes [18]. Nevertheless, despite the undeniable global obesity problem discussed so far, not everybody is obese, and in

healthy adults, BW and the amount of accumulated fat (i.e., the size of the body's fat stores) remain in fact amazingly stable. This is supposedly due to a potent physiological mechanism that maintains energy homeostasis. This mechanism allows for the matching of energy expenditure and energy intake over a long time. Its morphological substrate is a neuronal network linking different brain areas [39, 132]. In other words, the brain receives the information about the level of fat stored in the body through one or another signal and matches FI and energy expenditure adequately.

1.3.1 *Central control of eating*

One of the most important brain regions involved in the regulation of energy homeostasis is the hypothalamus [16, 130, 139]. The hypothalamus has the capacity to integrate the signals coming from the periphery, in particular the adiposity signals derived from adipose tissue, and it is also critical in responding to afferent signals from the gut and the brain stem [51]. The arcuate nucleus of the hypothalamus (ARC) contains several populations of neurons that are characterized by the expression of pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) or agouti-related peptide (AgRP) and neuropeptide-Y (NPY). Activation of the neurons that co-express NPY and AgRP increases FI and reduces energy expenditure, i.e., these neuropeptides are anabolic. Activation of the neurons that co-expresses CART and POMC decreases FI and increases energy expenditure, and these neuropeptides are therefore called catabolic [5, 14, 139, 166].

1.3.1.1 *POMC and CART neurones in the ARC*

POMC neurons release the neuropeptide alpha-melanocyte stimulating hormone (α-MSH), which inhibits FI through its capacity to bind to melanocortin receptors [72]. Pro-hormone convertase (PC) enzymes are required for POMC prohormone convertase PC1/PC3 to produce melano-cortins [74]. In case of α-MSH the MC4R receptor on the target neurons is required for it to inhibit FI [70]. Mice deficient in POMC have increased FI and, as a consequence, show weight gain [166]. In humans homozygous mutations in the POMC gene result in early onset of obesity, adrenal insufficiency and red hair pigmentation [83]. Also, polymorphisms of the MC4R are the most frequent mono-genetic contributors to overweight [58]. Subsets of POMC neurons are responsive to leptin. The POMC neurons that

express the leptin receptor are stimulated by leptin, and in leptin-deficient mice these neurons are inhibited [36].

The majority of POMC neurons co-express CART. Intracerebroventricular (ICV) administration of CART was shown to inhibit FI [82]. Blocking the flow of cerebrospinal fluid between the third and fourth ventricles eliminated this effect, suggesting that it originates from CART signaling in the hindbrain rather than the hypothalamus [2]. Unexpectedly, twice daily administration of CART into the ARC in rats for 1 week produced a 60% increase - rather than decrease - in FI [1,78,82]. This is in line with an experiment in which overexpression of CART in the ARC resulted in increased cumulative FI and BW gain [78], indicating that under certain conditions CART may have an orexigenic effect. Together these findings suggest that CART might be involved in at least two distinct circuitries where in one it may act as an anorectic and in the other as an orexigenic signal [139].

1.3.1.2 *NPY and AgRP neurones in the ARC*

NPY can act at five different receptors (Y1, Y2, Y3, Y4, and Y5) [132]. ICV injection of NPY in rats increases FI. The stimulatory effect of NPY on eating is mainly mediated through Y1 and Y5 receptors. Most NPY is co-expressed with AgRP [7, 17]. Deletion of NPY/AgRP in young mice was shown to reduce FI and BW [14, 54, 132]. Because NPY/AgRP neurons extensively project to the PVN, the DMN and the LHA interest focused on the capacity of these projections to mediate the enhanced FI [14]. ICV Injection of NPY and AgRP into the PVN produced a stimulation of FI [25, 139].

1.3.2 *Peripheral signals and gut peptides involved in energy homeostasis*

In previous paragraphs, I described some aspects of the hypothalamic circuitries in the regulation of energy homeostasis. I also mentioned that these central mechanisms are downstream of and activated or inhibited by peripheral signals. This raises the questions of 1) which are these peripheral signals, 2) how do they reach the brain, and 3) when and where do they emerge?

Many hormones and nutrient-related peripheral signals can influence energy homeostasis. Among them are satiating gut peptides such as peptide tyrosine tyrosine (PYY), glucagon-like peptide-1 (GLP-1), and cholecystokinin (CCK), which all can lead to meal termination, as opposed to FI stimulating peptides such as ghrelin. In addition to gut hormones, other

factors such as metabolic processes can also affect energy homeostasis and, in particular, FI [62]. As I cannot cover all the peripheral signals comprehensively within the scope of this thesis, I will mostly focus on signals related to my work:

Gut peptides: GLP-1 and CCK are satiation peptides that signal the brain together with neuronal signals generated by gastric distention. Mostly these two hormones, similar to gastric distension, trigger neural signals in afferent fibres of the vagus nerve, which project from the gut to the NTS in the caudal hindbrain [62].

Leptin signalling deficiency in the brain was shown to reduce the capacity of CCK to decrease FI, which resulted in an increase in meal size. Similar findings were reported for leptin and GLP-1 [99, 116]. Interestingly, the NTS and the ARC are reciprocally connected, both directly and indirectly. This interaction is important for the modulation of satiation signals by leptin, i.e., in essence for the modulation of eating by the amount of energy stored in the body [26, 51, 94]. In addition, leptin can modulate the satiation signals by acting on hindbrain leptin receptors [94, 132]

1.3.3 *Leptin action on the brain*

Every person has a different settling point of BW, which is the result of environmental factors acting upon a certain genetic background. As long as the environment or any other factor does not change, an energy balance will be established that may be maintained over a long time. As changes in BW of adult individuals are mainly due to changes in body fat, fat is supposed to be the critical variable that is kept relatively constant. Adipose tissue stores energy in form of lipids and has the capacity to release different compounds into the blood to inform the brain about the amount of energy stored, like the fuel gauge of a car. Leptin, a major secretory compound of adipose tissue, encoded by the ob gene, is the prototypical adiposity signal. In many conditions the plasma levels of leptin are approximately proportional to the size of the fat stores [27, 131]. By modulating energy intake and expenditure, leptin has the capacity to regulate the amount of fat stored in the body. In this context, low levels of leptin shut off all energy consuming activities and make any edible food attractive to prompt eating [23, 40, 94, 130, 132, 133, 162]. Leptin deficiency or leptin signalling deficiency causes an obese phenotype. In the case of leptin deficiency this obese phenotype can be fully reversed by leptin administration [159, 167]. Leptin can enter the brain and act on its receptor that is expressed in the

main neuronal populations involved in the regulation of energy balance in the hypothalamus, but also in other brain areas, in particular in the hindbrain [10, 21, 130]. A primary target of leptin in the hypothalamus is the hypothalamic ARC with its different sets of peptidergic neurons, secreting orexigenic neuropeptide Y and AgRP or the anorexigenic POMC (see above) [9, 36, 91, 132]. The central administration of leptin decreases FI and BW [9, 10, 23]. Leptin administration suppresses NPY and AgRP neurons and activates POMC and CART neurons [15, 23, 82, 162]. The overall effect of these actions is catabolic. In addition, as also mentioned before, leptin acts on PVN and LHA neurons, where it decreases the expression of orexin and melanin stimulating hormone (MSH) [79]. Fig. 1.2 (adapted from [132]) summarizes the hypothalamic signalling pathways downstream of leptin and insulin with increasing or decreasing body fat mass and with FI as a readout [132].

Leptin receptors are also expressed in the NTS, and 4^{th} ventricle leptin injection also reduces FI, whereas leptin receptor knockdown in the NTS was shown to increase FI [133]. Several neuronal populations in the NTS express functional leptin receptors, including Prepro-glucagon (PPG) neurons that produce GLP-1. Taking into account that both GLP-1 and leptin reduce FI, and that leptin depolarizes PPG neurons, PPG neurons can be regulated by leptin [50, 51, 55, 59].

Obese individuals commonly exhibit a reduced leptin responsiveness, which is often called leptin resistance. Leptin resistance could be due to either a compromised leptin transport across the blood brain barrier (BBB) or to decreased sensitivity of leptin receptors to leptin. Persistent high levels of circulating leptin can eventually cause leptin resistance [42, 145]. HFD feeding can induce obesity and leptin resistance [88, 91, 101]. Peripheral and central leptin resistance can be distinguished [24, 92]. Central leptin resistance was shown to occur in the hypothalamic ARC [91]. Signalling is also impaired in the VMN, DMN and PVN, whereas some neuron populations in the NTS appear to remain leptin sensitive [101, 130, 132, 145]. Whether pre-pro glucagon (PPG) neurons in the NTS are leptin sensitive or become insensitive, and under which conditions, is unclear and needs to be further investigated. Thus, within the CNS regionally selective leptin resistance seems to take place, and in general, central leptin resistance usually occurs later than peripheral resistance.

Insulin produced by β-cells of the pancreatic islets is also implicated in energy homeostasis regulation as an adiposity signal. Similar to leptin,

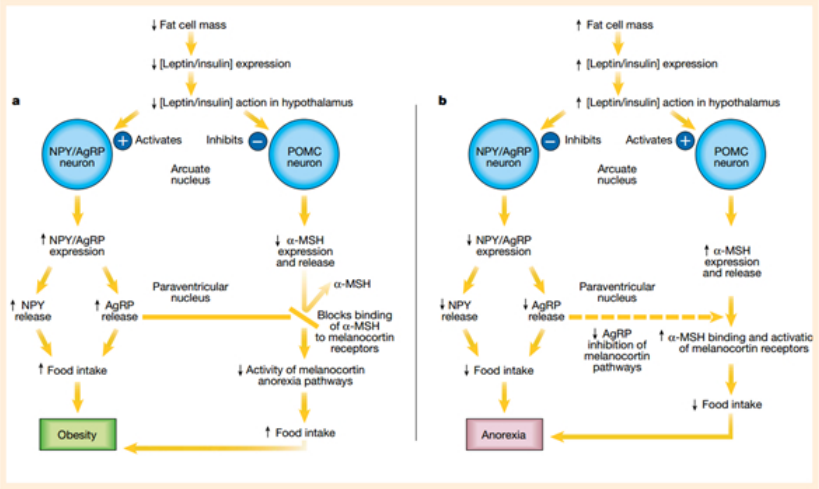

FIGURE 1.2: Schematic representation of signals regulating NPY/AgRP and POMC neurons in the context of food intake [132].

circulating insulin reflects the size of the body's fat stores and acts centrally to inhibit eating [170].

1.3.4 *Glucagon-like peptide*-1 *(GLP*-1*)*

GLP-1 is a 30 amino acid peptide, produced primarily in enteroendocrine cells in the intestine and in a distinct group of neurons in the NTS. In the intestine, GLP-1 is secreted by the open-type endocrine L-cells with a triangular shape, which are located in the basal lamina of the intestinal epithelium [19]. Their cytoplasmic processes reach the gut lumen. This way L-cells can sense digestion products of carbohydrates, lipids and proteins, i.e., the overall nutritional status in the gut lumen, and generate an appropriate response in terms of GLP-1 secretion [121,122]. The density of L-cells increases throughout the small and large intestines from oral to caudal, i.e., even the rectum contains a high density of L-cells [148,149].

GLP-1 is encoded by the pre-pro-glucagon gene, and the gene product undergoes post-translational processing by enzymes called pro-hormone convertases PC1, PC2 and PC3. These enzymes are encoded by the PscK1 gene [126–128, 169]. Importantly, the PC1-3 enzymes do not only process pro-glucagon and glucagon, but also proopiomelanocortin, proinsulin, and

proghrelin. Several studies have shown a relationship between the frequency of PCSK1 gene polymorphisms in the population and the increase in the susceptibility to obesity [11, 62].

After GLP-1 is released and diffuses from the basal lamina to the lamina propria, it is taken up into the capillaries. Some of the released GLP-1 also ends up in intestinal lymph. After its release, the enzyme dipeptidyl peptidase IV (DPP-IV) rapidly degrades GLP-1. DPP-IV is located on the surface of the endothelial cells lining the capillaries, and it can be cleaved to be released into the blood stream [31–34, 63]. The degradation of GLP-1 is so rapid that only about 25% of the released GLP-1 reach the hepatic portal vein. Substantial further degradation occurs in the liver [33]. As a result, only 10-15% of the initially secreted amount of GLP-1 reaches the systemic circulation and may eventually get to the pancreas and the brain. In addition to DPP-IV, there is an additional enzyme called neutral endopeptidase for which GLP-1 is a substrate [118]. The biological half-life of GLP-1 in the blood is therefore extremely short, i.e., only 1-2 min [150].

GLP-1 circulates in several forms, of which GLP-1 $(7-36)$ and GLP-1 $(7-37)$ are known to be active. There seems to be no specific difference between the functions of these two forms. The only known difference is that GLP-1 $(7-36)$ is more abundant than GLP-1 $(7-37)$ [62].

GLP-1 signals through the GLP-1 receptor (GLP-1R), which is a secretin receptor family class 2, a seven-transmembrane domain G-protein coupled receptor that is widely distributed in peripheral organs (pancreatic islets, heart, kidney, muscles, gastro-intestinal tract) and brain. The functions of GLP-1 for all these target tissues are not exactly known, but the most important known function of GLP-1 appears to be its incretin effect [115, 148, 161]. The term 'incretin' refers to the amplification of glucose-induced insulin secretion by a hormone secreted from the gastrointestinal tract. Experiments with the infusion of antagonists to GLP-1 and glucose-dependent insulinotropic peptide (GIP) in rats revealed that these two hormones are the most important incretins [47, 64, 65, 77, 151]. Interestingly, both hormones require the presence of increased plasma levels of glucose to stimulate insulin secretion. In other words, they enhance glucose-induced insulin release. Although GIP levels appear to be about 10-fold higher than GLP-1 levels, GLP-1 is reportedly more effective [45, 61, 102, 106, 152]. Activation of GLP-1 receptors does not only enhance glucose-induced insulin secretion, but also promotes β-cell insulin synthesis as well as β-cell proliferation, and it decreases β-cell apoptosis [62].

Besides its incretin effect, GLP-1 also affects nutrient absorption, primarily by slowing down gastrointestinal motility and gastric acid secretion. This presumably prolongs and thus enhances an eating-inhibitory signal from gastric vagal mechanoreceptors, which could contribute to the eating inhibition induced by GLP-1. The major satiating effect of endogenous GLP-1 is, however, mediated by vagal afferent GLP-1R [81, 111]. Under normal conditions, a direct central satiating effect of endogenous GLP-1 is unlikely, given the short biological half-life. Massively increased circulating levels of GLP-1 might however contribute to the eating-inhibitory and antidiabetic effects of bariatric surgery [87, 117].

1.3.5 *Role of PPG neurons and their possible regulators*

As mentioned above, GLP-1 is produced not only peripherally, but also centrally by a small population of neurons, the cell bodies of which are primarily located in the NTS. These neurons are characterized by the expression of PPG. In addition to the NTS, PPG neurons are also present in the medulla reticular formation and in the olfactory bulb. The PPG neurons in the olfactory bulb are, however, only local interneurons, i.e., most of the PPG projections in the brain originate from PPG neurons in the NTS [30, 73, 84, 103, 114, 124, 146, 153].

PPG neurons have direct projections to the following areas: the central autonomic area (CAA) , intermediolateral cell column (IML), spinal cord, arcuate nucleus (ARC), ventral tegmental area (VTA), dorsomedial hypothalamus (DMH), paraventricular nucleus (PVN), nucleus accumbens (NAc), and dorsal motor nucleus of the vagus (DMNX) [4, 37, 146, 153], as shown on Fig. 1.3 adapted from [146].

The highest density of PPG neuronal projections are found in the PVN and DMH. In addition, PPG neurons were shown to project to the NAc. The target areas of PPG neuronal projections usually express functional GLP-1R, whereas PPG neurons themselves lack GLP-1R [49, 84, 95, 114]. Little is known about which cell types in the NTS express GLP-1R. The lack of a proper GLP-1R antibody makes it very difficult to address this question.

Species differences between rats and mice were reported with respect to the function of PPG neurons and their regulatory mechanisms. For example, the PPG expression level in PPG neurons appears to be regulated by leptin in mice, but not in rats [68]. Importantly, also GLP-1R distribution in the brain differs between the two species, and it is still unclear, which

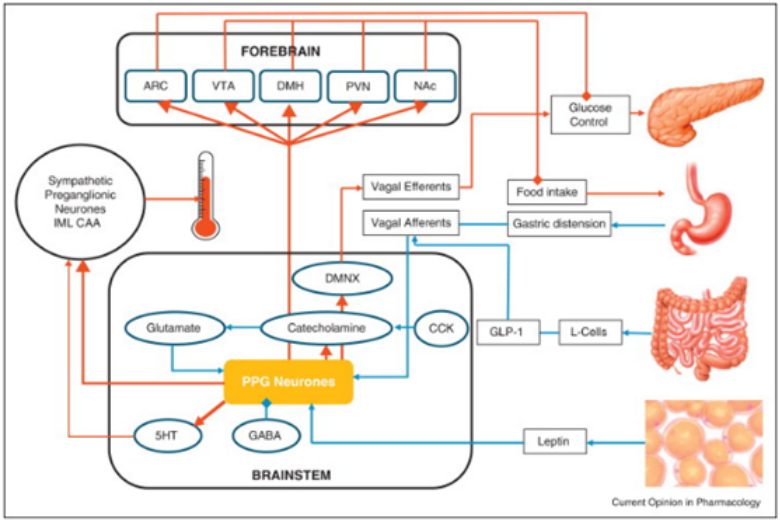

FIGURE 1.3: Schematic representation of brainstem PPG neuronal inputs and their projections. Brainstem PPG neurons receive inputs (blue) and sending outputs in (red) [146].

animal model more closely resembles the human situation. In mice, ICV injections of GLP-1R agonist have been shown to have many effects including a reduction of FI, improved glycemic control, hypothermia and neuroprotection [76]. In turn, the delivery of a GLP-1R antagonist into the 4th ventricle was shown to increase FI [57]. Studies in mice have also shown that PPG neurons can be activated by leptin, CCK, oxytocin, LiCl, and gastric distension [40, 46, 125, 146, 154]. Taking into consideration that some of these stimuli are considered to be 'aversive' this raises the possibility that the capacity of central GLP-1 to decrease FI might be partly though mechanisms involving induction of illness or nausea.

Several studies have addressed the role of PPG neurons using the designer receptor exclusive activated designer drug (DREADD) approach. Such activation of PPG neuronal terminals in the VTA reduced HFD intake compared to non-activated receptor expressing mice, and this was suggested to be achieved by reducing the synaptic drive onto mesolimbic dopamine neurons [156]. Although the study claimed to investigate the function of specific PPG neuronal activation, because they used Phox2b-Cre mice for DREADD virus injection, the employed method was not PPG specific; rather, it targeted a much broader neuronal population in the NTS.

So, there is still a need for studies specifically targeting PPG neurons in the future.

In rats, a lentivirus-mediated knockdown of NTS PPG neurons was performed to address the role of PPG [8]. This study reportedly achieved a 50% knockdown efficiency in NTS and a 30% knockdown efficiency at the PPG neuronal terminals in the PVN. Rats displayed hyperphagia, glucose intolerance and HFD-induced BW gain compared to scrambled shRNA injected control animals, indicating a physiological role of central PPG in energy homeostasis [8]. To my knowledge, PPG knockdown studies in mice have not been published so far. Also, whether central GLP-1 produced by PPG neurons has different effects from that produced in the periphery by enteroendocrine cells, or what the interactions between central and peripheral GLP-1 are, needs further investigation.

1.4 AIMS OF THE THESIS

GLP-1 is mainly produced in the L-cells of the small intestine and centrally by PPG neurons in the NTS. The production of GLP-1 in these two places raises the possibility that the sites of action as well as the functions of GLP-1 may differ depending on the origin of GLP-1. While peripheral GLP-1 and its effects have been extensively studied, less is known about the central GLP-1, and in particular about the regulation of GLP-1 producing PPG neurons. Several studies have shown that the production of GLP-1 in the gut is decreased in HFD-induced conditions of DIO [69]. One of the reasons for the dramatic antidiabetic and antiobesity effect of bariatric surgery supposedly are the substantially increased circulating levels of GLP-1 after surgery. Other studies suggest, that while peripheral GLP-1 decreases central PPG mRNA expression increases in obesity. The reasons and consequences of this central increase are unclear and require further investigations. Although the GLP-1 expression levels in the gut and the brain were not measured in the same experiments and can therefore not be directly compared, they still raise questions about this potentially divergent central and peripheral GLP-1 regulation.

As was briefly touched upon in Chapter 1 (introduction) previous studies have implicated NTS-derived GLP-1 in the control of energy intake and expenditure. In Chapter 2, I describe our investigations into central GLP-1 and PPG neuronal function in normal and obese conditions. In this context, our first aim was to verify previously published data on PPG mRNA upregulation upon HFD feeding and to gain further understanding with

respect to the relation between PPG mRNA levels and obesity onset. We therefore assessed central PPG mRNA levels during long-term HFD feeding in mice. The results suggested that leptin could be a possible regulator and the major reason of central PPG mRNA upregulation, which lead us to the second aim of this part of my thesis, i.e., to use leptin signaling deficient obese ob/ob and db/db mice to address this possibility. Because our results revealed that leptin has the capacity to modulate PPG levels, but is not the major reason of the upregulation of PPG, we further investigated the effect of leptin on PPG expression using chronic injections of a leptin receptor antagonist into the 4^{th} ventricle of DIO animals. To further examine the consequences of central PPG upregulation we used a LV-mediated central PPG KD in HFD-fed DIO mice to thoroughly characterize the induced phenotype. We also wanted to address the consequences of a modulation of PPG neurons. To this end we activated or silenced the PPG neurons in HDF-fed DIO mice using the DREADD-technology for further characterization.

In Chapter 3, finally, I focus on peripheral rather than central GLP-1. We cannulated the mesenteric lymph duct (MLD) to use lymph as a potentially better readout than hepatic portal vein (HPV) plasma for monitoring intestinal GLP-1 secretion. We equipped rats with MLD, HPV and IP catheters, allowing for parallel sampling of MLD lymph and HPV plasma as well as for peptide administration (the IP catheters). Animals were offered isocaloric HFD and LFD test meals for the monitoring of endogenous GLP-1 release. In addition, exogenous GLP-1 was injected at different doses to challenge the detection range and to see how an anorectic dose of GLP-1 would change the HPV and MLD GLP-1 concentrations. In addition to GLP-1, we measured insulin and metabolites, such as glucose and triglycerides, and how they evolved after the isocaloric HFD and LFD test meals.

In the Chapter 4, I summarize all findings and discuss their interpretations and limitations in a broader context and with respect to possible future studies.

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2

ROLE OF PPG NEURONS IN REGULATING ENERGY HOMEOSTASIS IN MICE

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2.1 ABSTRACT

Glucagon-like peptide-1 (GLP-1) produced in the nucleus tractus solitarii (NTS) is implicated in the control of energy intake and expenditure. Blocking central GLP-1 receptor (GLP-1R) signalling with a GLP-1R antagonist prevented diet-induced obesity (DIO) in mice, suggesting that the HFD-induced rise in brain GLP-1 levels contributes to DIO development. We here found that feeding mice a 60% HFD for 10 weeks increased NTS pre-pro-glucagon (PPG, GLP-1 precursor) mRNA expression compared to chow-feeding. Similarly, leptin deficient ob/ob mice showed elevated PPG mRNA expression in the NTS, which was reduced by leptin treatment, suggesting that the PPG gene is regulated by leptin.

Surprisingly, blocking leptin signalling in the 4^{th} ventricle using leptin receptor antagonist (LRA) decreased food intake (FI), body weight (BW), and NTS PPG mRNA levels in DIO mice. Furthermore, NTS PPG knockdown (PPG KD) using lentiviral-mediated RNA interference decreased BW gain and daily FI. The reduced FI in PPG KD mice was associated with a decrease in hypothalamic NPY expression. On the other hand, acute activation of PPG neurons using designer receptors exclusively activated by designer drugs (DREADD) technology also decreased short-term FI and energy expenditure (EE) in DIO mice, suggesting that a GLP-1-independent mechanism was responsible for the anorexigenic effect. Our results indicate that a chronic increase in central PPG levels with HFD feeding contributes to the obesity phenotype by modulating hypothalamic neural circuits involved in the control of energy intake and expenditure.

2.2 INTRODUCTION

Glucagon like peptide-1 (GLP-1) encoded by the pre-pro-glucagon gene (PPG) is produced peripherally by L-cells in the gut and centrally by a small population of neurons in the lower brain steam, i.e., in the nucleus tractus solitarii (NTS) and the medulla reticular formation [1]. The PPG gene does not only encode for GLP-1 and glucagon, but also for other hormones. Posttranslational modification of pre-pro-glucagon by prohormone convertases (PC1, PC2, PC3) leads to GLP-2, oxyntomodulin (OXM), intervening peptide-1(IP-1) and glicentin (GCG peptides) [2–4].

Gut-derived GLP-1 enhances glucose-induced insulin secretion, inhibits gastric emptying and reduces FI [5, 6]. GLP-1 receptor (GLP-1R) agonists are currently approved for the treatment of type-2 diabetes (T2D), and one of them (liraglutide) was recently also approved for the therapy of obesity [7, 8]. While the role of peripheral GLP-1 is well established, less is known about the role of centrally produced GLP-1. Because systemically applied GLP-1R agonists can directly activate GLP-1R in the hypothalamus and in the hindbrain [9, 10], understanding GLP-1 signalling in the brain will be crucial for developing a GLP-1R agonist-based strategy to combat obesity.

In previous studies ICV injections of GLP-1R agonists reduced FI and glycemia, had a neuroprotective effect and produced hypothermia in mouse and rat models [1,6,11–17]. Furthermore, activation of PPG neuronal terminals in the ventral tegmental area (VTA) using a chemogenetic (DREADD) approach reduced consumption of a high-fat diet (HFD) compared to control treatment through a reduced synaptic drive onto mesolimbic dopamine neurons [18, 19].

PPG neurons are activated by various signals including cholecystokinin (CCK), oxytocin, lithium chloride (LiCl), gastric distension, and leptin [1]. In fact, PPG neurons express functional leptin receptors (LepR), and they are depolarized upon the administration of leptin [20]. Fasting, which reduces circulating leptin levels, results in a downregulation of central PPG mRNA expression, whereas leptin injection can restore the PPG levels [11]. Less is known, however, about the role of leptin signalling in PPG regulation in obesity, and this needs further investigations.

PPG neurons directly project to different brain areas including the hypothalamus. The arcuate nucleus of the hypothalamus (ARC) contains neuropeptide Y (NPY)/agouti-related protein (AgRP) and pro-opiomelanocortin (POMC)/cocaine- and amphetamine-related transcript (CART) neu-

rons that are considered major controllers of FI [21]. Upon stimulation, NPY/AgRP neurons promote, whereas CART/POMC neurons inhibit, eating [22–29]. Under normal feeding conditions GLP-1R agonist was shown to directly stimulate POMC/CART neurons via GLP-1R, but it inhibits NPY/AgRP neurons via an indirect mechanism [9]. It is, however, unknown how endogenous central GLP-1 affects these neurons in obesity.

Previous studies have shown that chronic ICV administration of the GLP-1R antagonist exendin-9 for 4 weeks, which blocks central GLP-1R signalling, prevents the development of DIO [10], raising the possibility that the increase in central GLP-1 levels might contribute to the development of obesity. To investigate the role of central GLP-1 in DIO, we here characterized hindbrain PPG mRNA expression in several obese mouse models and examined the effects of leptin signalling in PPG gene regulation. We also addressed the role of PPG overexpression in DIO mice by knocking down PPG mRNA using lentiviral mediated RNA interference. In addition, we used a chemogenetic tool to manipulate PPG neuronal activity and evaluated the consequences of this manipulation for FI, BW and energy expenditure (EE).

2.3 MATERIALS AND METHODS

2.3.1 *Animals and housing*

All experiments were approved by the Canton of Zurich Veterinary Office. The animals were kept in type 2 Macrolon cages in an air conditioned animal holding room ($21 \pm 2\,^{\circ}C$ and $50 \pm 5\%$ humidity) maintained on a 12-hour light/12-hour dark cycle (light on at 7 : 00 am). All animals had ab libitum access to water and food, which was regular chow (CD, Kliba 3436, ProvimiKliba NAFAG, Kaiseraugst, Switzerland) unless mentioned otherwise.

All mice were bred in our animal facility: WT mice: Initial C57BL6J breeders were obtained from Charles River (Charles River, Sulzfeld, Germany). Glu-Cre mice: Initial breeders were obtained from Fiona Gribble/Frank Reimann (University of Cambridge Metabolic Research Laboratories, Cambridge, United Kingdom). The Glu-Cre mice express the Cre recombinase under the glucagon promoter, the mice were bred Cre-heterozygous to avoid possible Cre-induced toxic effects. Glu-YFP mice: Initial breeders were also obtained from Fiona Gribble/Frank Reimann. The Glu-YFP mice express the YFP fluorescent protein under the glucagon promoter.

The mice were bred as heterozygous. ob/ob mice: Initial breeders were obtained from Charles River (Charles River, JAX™Mice Strain,B6.Cg-Lepob/J. Sulzfeld, Germany). db/db mice: Initial breeders were obtained from Charles River (Charles River, JAX™Mice Strain,B6.BKS(D)-LepRdb/J. Sulzfeld, Germany).

2.3.2 *DIO studies*

For the DIO studies, animals were switched to HFD (60 kJ% fat, SSNIFF E15742-34 EF D12492 (I) mod) at the age of 8 weeks. The duration of HFD feeding is indicated for each experiment (see below).

2.3.3 *Surgeries*

Animals were anesthetized by IP injections of an anesthetic mixture consisting of 5 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany) and 86 mg/kg ketamine (Ketasol-100; Dr. E. Gräub, Bern, Switzerland). Microinjections of all viruses were performed with glass pipet tips bilaterally (50 µm) into the caudal NTS (coordinates: 0.1 mm rostral, 0.5 mm lateral to obex and 3.5 mm ventral to surface). An appropriate volume of viral solution was injected into each side using the Picospritzer III injector (Parker Hannifin, Precision Fluidics Division, East Pine Brook, NJ REF: 07058 973-575-4844). The wound was sutured using 6.0 surgical sutures (Ethicon Coated VICRYL REF: V384). After surgery the animals were subcutaneously (SC) injected (1 ml/kg BW) with a mixture of 20 mg/kg sulfadoxin and 5 mg/kg trimethoprim (Borgal 24%, Intervet, Shering-Plough Animal Health, Kenilworth, NJ) and Carprofen (Norocarp, UFAMED AG, Sursee, Switzerland), resulting in a final dose of 5 mg/kg BW. Borgal was injected only on the surgery day, whereas Norocarp was injected for 3 days post-surgery. To ensure sufficient expression of the virus, the experiments began 2 weeks post-surgery.

2.3.4 *PPG LV-shPPG knockdown*

To achieve PPG knockdown, stereotactic injections of lentiviral-mediated PPG shRNA was performed in both 60% HFD and CD-fed WT mice. Adult WT mice were injected with pLKO.1-puro vectors expressing turbo green fluorescent protein (GFP) and a U6 promoter driven shRNA targeting the mouse PPG mRNA sequence (LV-shRNA) or nontarget shRNA (LV-

control)(Sigma-Aldrich) as control. The efficiency of the PPG knockdown was assessed in the mouse enteroendocrine Glu-Tag cell line expressing PPG. Post-transfection cells were sorted by FACS and knockdown efficiency was checked using Q-PCR. Selected targeting (LV-shPPG) as well as control (LV-control) lentiviral particles were produced in human embryonic kidney cells (HEK-293-T) using the pMD2.G and psPAX2 plasmids (gifts from D. Trono, École Polytechnique Fédérale de Lausanne; cat. no. 12259 and 12260; Addgene) and concentrated using 8% PEG6000 (Millipore) and resuspended in PBS. Virus was injected bilaterally as described in 'animal surgeries' above at a volume 300 nl per site.

2.3.5 *DREADD activator / silencer virus injection*

Glu-Cre mice were used to investigate the behavioural consequences of manipulating the PPG neurons with the designer receptors exclusively activated by designer drug (DREADDs) system upon clozapine-N-oxide (CNO) stimulation. CNO was always dissolved freshly from powder as follows: 0.5 mg of CNO were resuspended with 10 µL of DMSO, which was followed by the addition of 5 ml PBS for a final injection of 0.3 mg/kg of mice BW. We used hM3Dq (AAV5-hSyn-DIO-hM3Dq-mCherry) and hM4Di (AAV5-hSyn-DIO-hM4Di-mCherry) DREADD (North Carolina UNC vector Core) that are Cre-dependent. The virus was used according to the manufacturer's instructions.

2.3.6 *Leptin receptor antagonist (LRA) injection*

For the LRA preparation, lyophilized mouse super-active leptin antagonist (SMLA) was purchased from Protein Laboratory (Rehovot, Israel, Cat. #SLAN-1) and stored at −20 °C. LRA was dissolved in double distilled water and mixed gently resulting in a final stock concentration of 5 µg/µL. For the LRA injection, a 4 mm long plastic cannula (22G) with the injector protruding 1 mm was stereotaxically positioned into the 4^{th} ventricle of Glu-YFP HFD-fed mice (coordinates: 2.4 mm AP, 0 mm ML, −4.0 mm DV to the Lambda). The cannulas were fixed to the skull using 2 screws and dental cement. Starting one week after surgery, sham injections were performed through the cannula for 3 days followed by LRA treatment. LRA injections (0.5 µg/µL injection volume 0.5 µL) were performed for 7 days twice daily 1 h prior to the light or dark phase.

2.3.7 *Oral glucose tolerance test (OGTT)*

Mice were food-deprived for 6 h with ad libitum access to water from the beginning of the dark phase. A baseline measurement was taken and an oral bolus of 20% glucose solution (Sigma – Aldrich CAS: $50-99-7$) in water was given through gavage at the dose of 2 g/kg BW). Blood samples for glucose were taken from the tail vein at baseline and $15, 30, 60, 90$, and 120 min after the gavage. Glucose levels were measured using the commercially available glucometer and corresponding glucose strips (Aviva Roche, Accu-Check).

2.3.8 *mRNA level quantification of other genes*

For quantitative assessment of other genes in addition to PPG, extracted RNA was first subjected to reverse transcription–PCR as described before. This was followed by quantitative PCR using commercially available SYBR green (Fast SYBR Green Master Mix Applied Biosystems by ThermoFisher Scientific Cat. # 4368813) according to the manufacturer's recommendations using the following primers:

NPY
Forward: 5′-CCG CTC TGC GAC ACT ACA T-3′
Reverse: 5′-TGT CTC AGG GCT GGA TCT CT-3′

POMC
Forward: 5′-AGT GCC AGG ACC TCA CCA-3′
Reverse: 5′-CAG CGA GAG GTC GAG TTT G-3′

CART
Forward: 5′-CGA GAA GAA GTA CGG CCA AG-3′
Reverse: 5′-CTG GCC CCT TTC CTC ACT-3′

AgRP
Forward: 5′-CAG GCT CTG TTC CCA GAG TT-3′
Reverse: 5′-TCT AGC ACC TCC GCC AAA-3′

PC1
Forward: 5′-CCA AAG TTG GAG GCA TAA GAA TG-3′
Reverse: 5′-GTC TGT GTA GCC ATC ACA GTC A-3′

PC2
Forward: 5′-GCC GTG TTT GCA TTG GCT TT-3′
Reverse: 5′-GCA CAG TCA GAT GTT GCA TGT-3′

PC3
Forward: 5′-ACT TGG TCA GCC TCC CAT AGT TGT-3′
Reverse: 5′-TGT TAG CTG CCA GAC CAC ATG ACT-3′

GAPDH
Forward: 5′-ACCACAGTCCATGCCATCAC-3′
Reverse: 5′-CACCACCCTGTTGCTGTAGCC-3′

2.3.9 *Mouse perfusion*

Mice were anesthetized using 50 µL of pentobarbital and then transcardially perfused at a speed 20 ml/min with PBS for 1 min. Thereafter fixation was done with a 4% PFA solution containing 15% picric acid for 3 min. The brain was taken out from the skull and placed in a vial with 4% PBS/15% picric acid for $6-9$ h followed by incubation in citric buffer (pH 4.5) containing Na_2HPO_4 dihydrat (0.2 M) and citric acid (0.1 M) at RT for at least 6 h. For the subsequent antigen retrieval the brain was heated in 80 ml citric buffer (RT) in the microwave (650 W, 80% power) for 85 seconds. Directly after the microwaving the brain was placed in RT PBS for 1 min, than in 4 0 °C PBS for 1 min, followed by 10% sucrose solution for 3 min. Finally, the brains were incubated in 30% sucrose solution for 72 hours. When the brains sunk to the bottom of a vial they were frozen and stored at -80 °C.

2.3.10 *Immunohistochemical staining*

Perfused and antigen retrieved frozen brains were sliced into 25 µm slices and stored in cryo protectant at -20 °C. For the staining, brain slices were rinsed with phosphate buffered saline (PBS) for 3 x 10 min. Brain slices were then blocked using blocking solution consisting of 2% donkey serum and PBST (0.3% Triton-X 100 in PBS) for 30 min. Thereafter, the slices were incubated with a primary antibody (1 : 200 rabbit anti-PSTAT-3-antibody) in 2% blocking-solution for 48 h at 4 °C. The brain slices were washed 3 times with PBS for 10 min followed by incubation for 1 h at RT with a secondary antibody (biotinylated donkey anti rabbit antibody, 1 : 5000 in PBST). After washing with PBS, the slices were placed for 20 min at RT in a solution composed of PBS, biotinylated tyramide-solution (BTS) (5 µL per mL PBS) and 3%-hydrogen-peroxide (3.3 µL per mL PBS). After washing, brains were incubated in 1 : 200 streptavidin 593-conjugate solution for

3 h at 37 °C. The brain slices were mounted on slides, air dried, fixed with glycine buffer and finally cover-slipped. Using a fluorescence microscope (CW Scope A.1, Zeiss, Oberkochen, Germany), fluorescence positive cells were visualized and photographed with a digital camera (Axiocam MRX, Zeiss, Oberkochen, Germany).

2.3.11 *Phenomaster study*

Mice were single housed for indirect calorimetry using PhenoMaster/ LabMaster metabolic cages (TSE systems, Bad Homburg, Germany). BW was manually measured daily and used to normalize EE. Data are shown as average of 2 days divided in 6 h bins.

2.3.12 *Statistical analysis*

For all statistical analyses StatView version 5.0 (Distributor: SAS institute, cary NC27513, USA. www.statview.com) was used. For statistical comparisons, an analysis of variance (ANOVA) was performed, followed by Bonferroni post-hoc tests. Significance was set at $p < 0.05$. All figures were plotted using GraphPad Prism version 6.0.

2.4 RESULTS

2.4.1 *HFD exposure increases central PPG mRNA expression in mice*

As described previously [10, 30, 31], long-term HFD exposure increased BW compared to standard chow diet (CD) (Fig. 2.1a, 10 weeks $p < 0.05$, 18 weeks $p < 0.05$). Consistent with this BW phenotype, HFD feeding increased plasma leptin levels (Fig. 2.1b, 10 weeks $p < 0.001$, 18 weeks $p < 0.0001$). Two weeks of HFD feeding did not yet result in a significant BW or PPG mRNA change compared to the CD group, indicating that PPG mRNA expression was not altered by the high fat content of the diet. Instead, the increase in PPG mRNA expression observed after 10 weeks was associated with the rise in BW and leptin levels (Fig. 2.1c, 10 weeks $p < 0.0001$, 18 weeks $p < 0.001$). Furthermore, 10 weeks of HFD feeding increased the number of cells expressing YFP under the PPG promotor (Glu-YFP mice), indicative of increased promotor activity for PPG gene expression (Fig. 2.1 d, e). These data suggest a positive correlation between BW, presumably fat mass, and central PPG mRNA production.

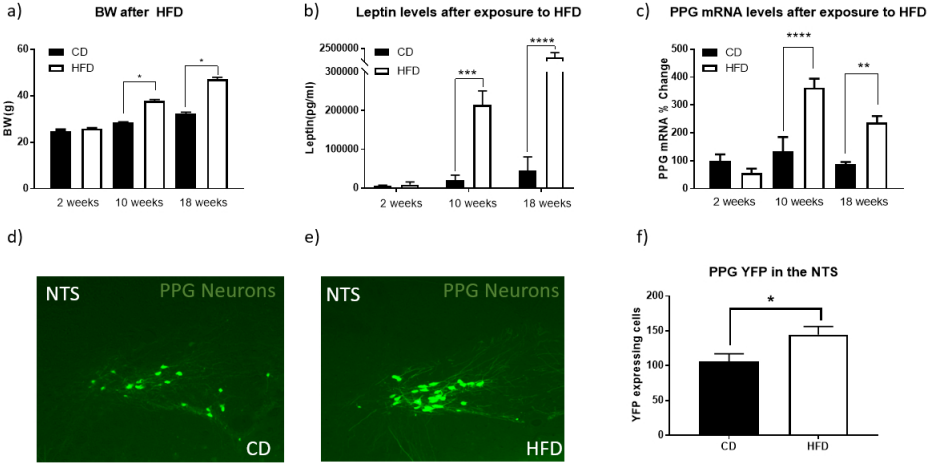

FIGURE 2.1: Influence of long-term HFD feeding on BW, circulating leptin levels, PPG mRNA levels and number of PPG neurons in WT mice. All measurements taken after 2, 10, and 18 weeks of HFD feeding. BW ($n = 14$): two-way ANOVA $p < 0.05$, with diet effect $*p < 0.05$; b) leptin levels ($n = 6$): two-way ANOVA $p < 0.0001$, with diet effect $***p < 0.0005$, $****p < 0.0001$; c) NTS PPG mRNA level ($n = 6/9$): two-way ANOVA $p < 0.0001$, with diet effect $**p < 0.005$, $****p < 0.0001$; d) YFP expression (green) under the PPG promotor, indicating PPG neurons in the NTS of Glu YFP mice fed CD; e) PPG neurons in the NTS of Glu YFP mice fed a HFD for 10 weeks; and f) quantification of YFP expressing PPG neurons in CD vs. HFD mice ($n = 7/9$): Student t-test $*P < 0.05$).

2.4.2 *Leptin signalling deficiency increases central PPG mRNA expression in mice*

PPG neurons express the leptin receptor [20]. To understand the role of leptin receptor signalling in PPG expression, we first confirmed signal transducer and activator of transcription- 3 (STAT3) activation in PPG neurons after leptin (IP, 2 µg/kg BW) administration in Glu-YFP mice (Fig. 2.2a). Our qualitative assessment revealed the presence of functional Leptin receptors in PPG neurons.

Interestingly, hindbrain PPG mRNA levels in leptin deficient ob/ob mice were higher than in their WT littermate controls (Fig. 2.2b, $p < 0.0001$). Leptin administration rapidly decreased PPG mRNA expression in ob/ob mice, whereas it did not affect PPG mRNA expression in WT mice, suggesting that PPG mRNA overexpression is part of starvation signals that contribute to hyperphagia in the leptin deficiency model. These data also suggest that, in the presence of endogenous leptin, additional exogenous leptin does not affect PPG mRNA expression.

Similarly, PPG levels in db/db mice were higher ($p < 0.001$) than in their WT littermates (Fig. 2.2c). Leptin injection did however not result in a reduction of PPG mRNA levels in db/db animals, consistent with the role of leptin signalling through the leptin receptor in PPG mRNA regulation.

2.4.3 *Chronic LRA injection into the 4th ventricle triggers BW loss*

We hypothesized that leptin, which gradually increases during the HFD feeding, has a regulatory effect on PPG neurons. Because PPG neurons have functional leptin receptors, excess leptin might cause their constant activation or resistance. In order to study the influence of leptin, we fed mice HFD for 18 weeks to cause DIO, then performed 4^{th} ventricle cannulation surgery to deliver LRA locally in close proximity of PPG neurons. After recovery, the mice received twice daily ICV LRA injections for 7 days to block the leptin receptors. LRA injections resulted in a significant BW decrease compared to the vehicle (VEH) injected control group (Fig. 2.2 e, $p < 0.05$). We then generated a separate batch of animals with 4^{th} ventricular cannulae to perform an acute LRA injection and sacrificed them after 30 min for measuring central PPG mRNA levels. We found a 30% decrease in PPG mRNA levels in the LRA group compared to the VEH control group, although the difference did not reach statistical significance (Fig. 2.2 f). These data indicate that blocking leptin receptor signalling in

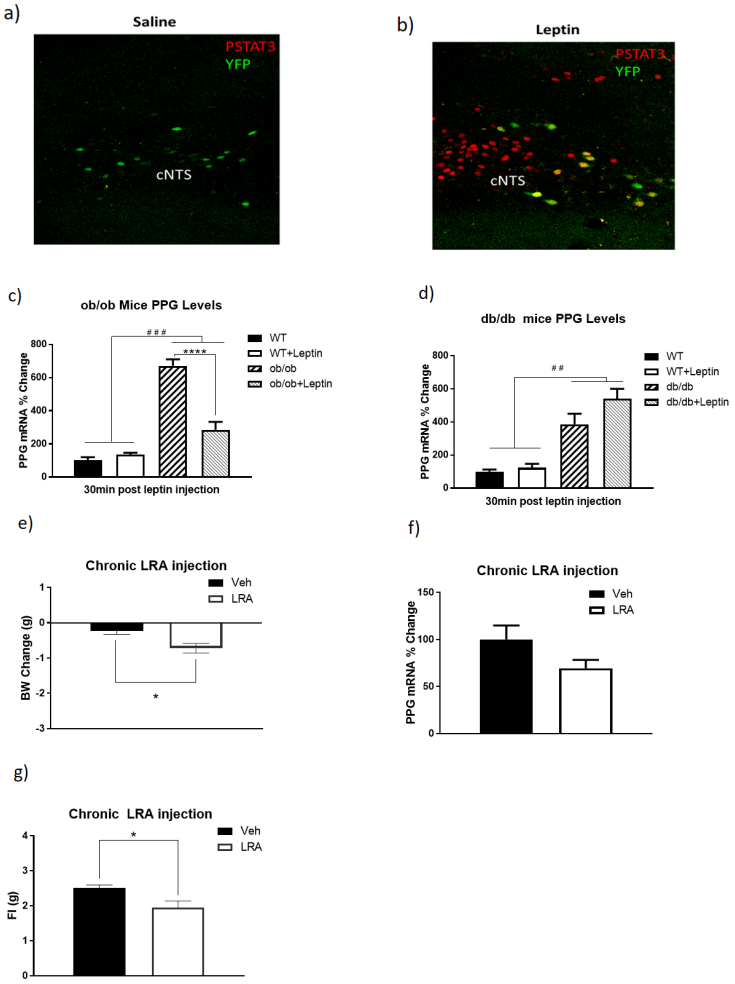

FIGURE 2.2: Leptin regulation of PPG neurons: PSTAT-3 staining (red) in GLU-YFP mice after: a) saline injection and b) 2 µg/kg IP leptin injection, c) PPG mRNA levels in ob/ob mice kept on CD; $n = 8$, Two-way ANOVA $p < 0.0001$, with treatment effect $****p < 0.0001$, group effect $***p < 0.0005$ d) PPG mRNA levels in db/db mice kept on CD; $n = 8$, Two-way ANOVA $p < 0.0001$, with treatment effect NS and group effect $**p < 0.005$, e) BW measurement of DIO mice after chronic (twice a day LRA injections for 7 days) kept on HFD ($n = 7$ Student t-test $p < 0.05$), f) PPG mRNA levels in DIO WT mice 30 min post LRA injection ($n = 7$ Student t-test $p < 0.05$), g) FI measurement in DIO mice after chronic LRA injections ($n = 7$ Student t-test $p < 0.05$).

the hindbrain results in a decrease in FI, BW, and PPG levels, suggesting that excess leptin signalling in the hindbrain can contribute to obesity.

2.4.4 *PPG mRNA knockdown decreases FI and ameliorates BW gain in DIO mice*

LV-shPPG or LV-control expressing GFP was injected bilaterally into the NTS in DIO mice (Fig. 2.3a). The LV-shPPG injection led to a 30% reduction in PPG mRNA levels in the NTS, compared to the scrambled LV-control (Fig. 2.3b, $p < 0.05$). The LV-shPPG injection reduced BW gain compared to LV-controls (Fig. 2.4c, $p < 0.05$). The PPG mRNA knockdown also resulted in a decrease in FI during the dark, but not during the light phase, compared to its LV control, (Fig. 2.3d, $p < 0.05$). Consequently, 24 h FI in LV-shPPG mice was also lower than in LV-controls (Fig. 2.3d, $p < 0.05$). The NTS PPG mRNA knockdown, however, did not significantly change fat or lean mass. (Fig. 2.4e). LV-shPPG and LV-control mice also showed similar glucose profiles during an OGTT (Fig. 2.3f).

2.4.5 *PPG mRNA knockdown reduces hypothalamic NPY expression*

PPG neurons project to major hypothalamic areas involved in FI and BW regulation. To evaluate if PPG mRNA knockdown had any influence on the hypothalamic expression of genes involved in FI control, we checked hypothalamic NPY, POMC, CART and AgRP mRNA expression levels as well as NTS expression levels of NPY, POMC and CART. LV-shPPG mice, compared to LV-controls, showed a significant decrease in NPY mRNA expression levels in the hypothalamus (Fig. 2.4d $p < 0.05$), whereas the mRNA levels of CART, AgRP and POMC were unchanged (Fig. 2.4a, b, c). On the other hand, LV-shPPG mice showed no change in NPY, CART, and POMC mRNA expression levels compared to LV-controls in the NTS (Fig. 2.4e, f, g). These data suggest that PPG neurons control FI by modulating hypothalamic NPY neurons.

2.4.6 *PPG mRNA knockdown does not affect PC enzyme levels in NTS*

PPG mRNA is post-translationally modified by PC1/2/3 enzymes, resulting in different post-processing products. We determined whether the PC1, PC2 and PC3 levels in the NTS differed between CD and HFD-fed mice and as a result of the LV-shPPG knockdown. Our findings revealed no sig-

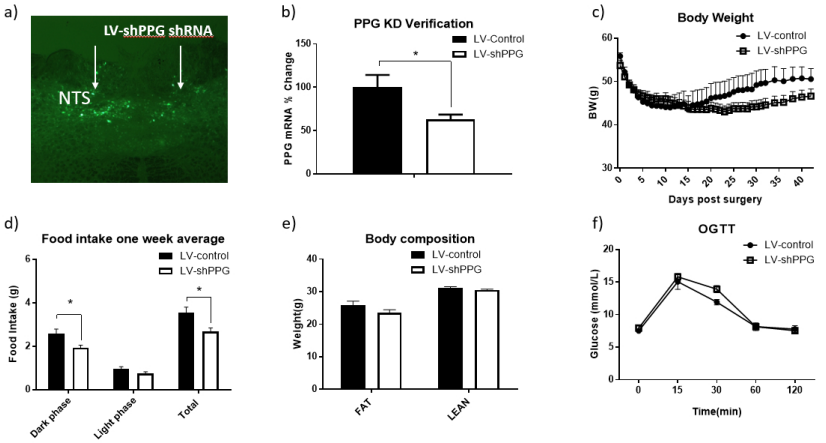

FIGURE 2.3: Viral-mediated PPG mRNA knockdown (LV-shPPG versus LV-control) in the NTS (PPG neurons) of DIO mice: WT mice were kept on a HFD for over 22 weeks after PPG KD was performed a) Injection site visualizing viral LV-shPPG shRNA expressing GFP in the NTS of DIO mice kept on HFD, representative image b) PPG mRNA levels in the NTS after LV-shPPG injection versus LV-control measured after all experiments were performed and mice were sacrificed ($n = 8/12$ Student t-test $p < 0.05$) c) BW measurement over 40 day post-surgery, LV-shPPG injection versus LV-control, d) FI measurement two weeks post-surgery, LV-shPPG injection versus LV-control, e) Echo-MRI quantification of body composition (lean and fat mass), performed 30 days after surgery, LV-shPPG injection versus LV-control, f) Glucose tolerance test, performed more than 3 weeks post-surgery, LV-shPPG injection versus LV-control.

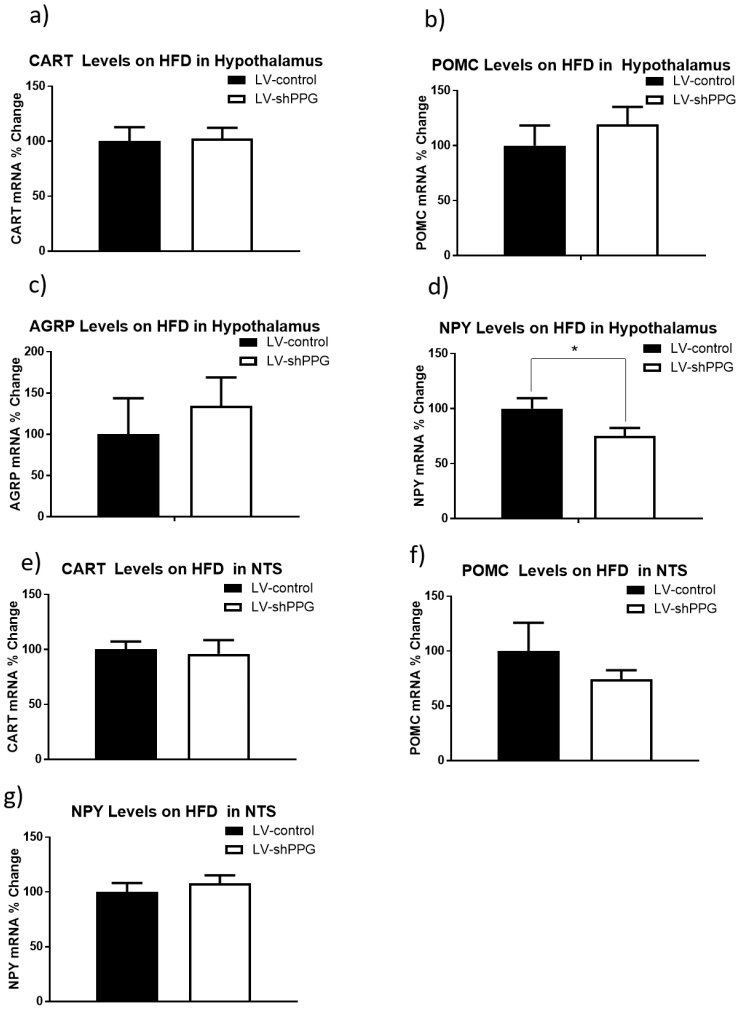

FIGURE 2.4: Effects of PPG knockdown on hypothalamic and NTS neuropeptide mRNA expression in DIO mice kept on HFD: WT mice were kept on HFD for 22 weeks; then PPG KD virus was bilaterally injected; 2 month post-injection mice were sacrificed and mRNA for next peptides were measured, Cocaine and amphetamine regulated transcript (CART), Pro-opiomelanocortin (POMC), Agouti-related protein (AgRP), Neuropeptide $Y(NPY) * p < 0.05$ in Student t-test ($n = 8/12$). a) Hypothalamic CART, b) Hypothalamic POMC, c) Hypothalamic AGRP, d) Hypothalamic NPY, e) Hindbrain CART, f) Hindbrain, POMC g) Hindbrain NPY.

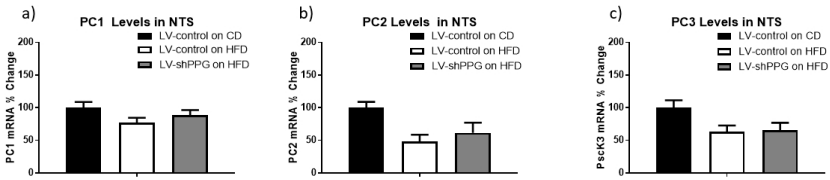

FIGURE 2.5: mRNA expression level of PPG processing enzymes Pro-protein convertase 1, 2, 3 in DIO WT mice after PPG mRNA knockdown (LV-PPG versus LV-control injection) in hindbrain. a) PC1, b) PC2, c) PC3

nificant difference among these three groups (Fig. 2.5). There was a trend towards a decrease in PC enzymes in HFD-fed compared to CD-fed mice. This lack of a change in PC enzymes makes it very unlikely that the processing enzymes could have shifted the PPG mRNA products in favor of one or the other post-processing compounds. Because PC enzymes (PC1, PC2 and PC3) process PPG gene products into GLP-1 and other peptidesa shift in the ratio of PC enzymes in the hindbrain during HFD feeding might have caused the upregulation of PPG mRNA. HFD-fed mice however showed only a trend towards a decrease in the expression of these processing enzymes compared to CD-fed mice, whereas PPG (LV-shPPG) KD had no effect on their levels compared to LV-controls.

2.4.7 *Acute PPG neuronal activation and silencing using hM3Dq and hM4Di DREADD has opposite effects on food intake*

To gain a better understanding of the function of PPG neurons, we used a designer receptor exclusively activated by a designer drug (DREADD) to specifically activate or silence PPG neurons. After viral vector delivery into the NTS, the DREADD receptor can be expressed upon Cre recombinase in Glu-Cre mice. Activation or silencing of neurons was achieved by peripheral injection of the synthetic ligand clozapine N-oxide (CNO). The human M3 muscarinic receptor hM3Dq was expressed in PPG neurons of Glu-Cre mice and chemo-genetically stimulated by CNO injection to achieve neuronal activation. To achieve PPG neuronal silencing, the human M4 muscarinic receptor hM4Di was expressed in PPG neurons of Glu-Cre mice and chemo-genetically stimulated by CNO injection. To examine the effect of PPG neuronal activation on eating in DIO mice, we stimulated PPG neurons with the transfected hM3Dq receptor with CNO

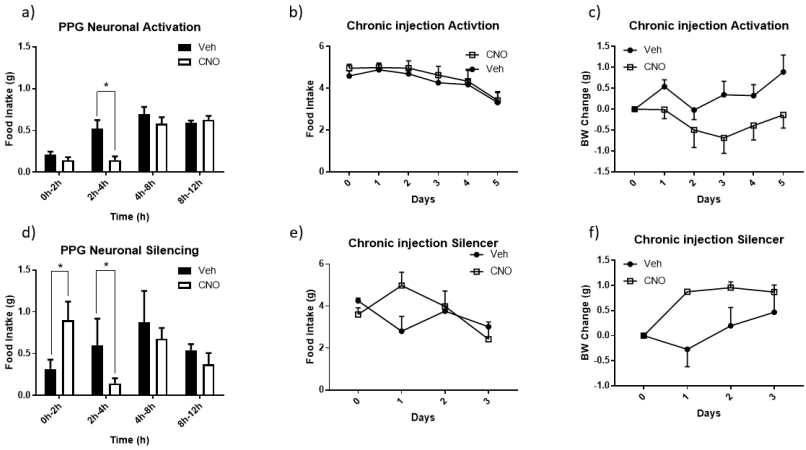

FIGURE 2.6: FI and BW after DREADD-mediated PPG neuronal activation or silencing in DIO mice: Glu-Cre mice were kept on a HFD for over 18 weeks, DREADD injection was performed in all animals and animals were allowed to recover for more than 2 weeks prior to experiments. CNO (injected at a dose 0.3mg/kg of BW) was compared to VEH control. Injections were performed either acutely i.e. one time or chronically i.e., every 12 hours $10-20$ min prior to light/dark onset: a) FI after acute CNO activation acutely, $n=6$, two-way ANOVA $p<0.05$, with treatment effect $*p<0.05$ b) FI after chronic activation, , $n=6$, c) BW after chronic activation, $n=6$, two-way ANOVA , no significance d) FI after acute silencing, $n=4$, two-way ANOVA , no significance e) FI after chronic silencing, $n=4$, two-way ANOVA , no significance f) BW after chronic silencing $n=4$ two-way ANOVA , no significance.

and measured HFD intake. CNO reduced the amount of HFD consumed compared to the VEH group (Fig. 2.6a, $p<0.05$). The eating-inhibitory effect of CNO lasted 4 h. Next, we examined whether PPG neuron silencing had an opposite effect on FI. To do so, we injected CNO in hM4Di receptor transfected mice; CNO increased the amount of HFD consumed within 2 h post-injection, compared to the VEH group. But, the mice compensated for this initial hyperphagia by consuming significantly less food afterwards (Fig. 2.6d, 0 – 2 h $p<0.05$, and 2 – 4 h $p<0.05$).

2.4.8 *Chronic PPG neuronal activation and silencing with hM3D1 and hM4Di DREADD increases and decreases body weight, respectively*

To study the consequences of a chronic PPG neuronal activation, hM3Dq was expressed in PPG neurons of Glu-Cre mice and chemo-genetically stimulated by CNO injection. CNO was IP injected twice daily for 5 days. This resulted in a significant reduction of BW in CNO injected animals compared to the VEH group (Fig. 2.6c, $p < 0.05$), whereas FI did not differ between the two groups during the injection period (Fig. 2.6b), suggesting that the BW loss of CNO injected mice was due to an increase in EE. To study the consequences of chronic PPG neuronal silencing, hM4Di was expressed in Glu-Cre mice. CNO was injected twice daily for 3 days in mice with the PPG neuronal silencer. This resulted in a significant increase of BW in CNO injected animals compared to the VEH group (Fig. 2.6f, $p < 0.05$). There was a significant increase in 24 h FI after the first day of CNO injection (Fig. 2.6e, $p < 0.05$), but this effect did not persist on the remaining days. To assess EE after PPG neuronal activation or silencing, mice were placed in a phenomaster system for indirect calorimetry measurements, including respiratory exchange ratio (RER), heat production, and locomotor activity. Chronic PPG neuronal activation led to a decrease in RER during the dark phase (corresponding to the FI reduction) and to an increase during the light phase (Fig. 2.7a). There was an increase in heat production in both the dark and light phase (Fig. 2.7b), consistent with the observed BW loss. No group difference in activity levels was found (Fig. 2.7c). Ironically, chronic PPG neuronal silencing also caused an increase in heat production with no change in RER and activity (Fig. 2.7d-f).

2.5 DISCUSSION

Peripheral GLP-1 is produced in L-cells in the small intestine and is released in response to nutrient-derived signals following meal ingestion [6]. GLP-1 acts on GLP-1 receptors (GLP-1R) of the vagus nerve, as well as directly on pancreatic β-cells, to enhance glucose-stimulated insulin secretion [6, 32, 33]. Endogenous GLP-1 also slows gastric empting and reduces FI [2,6,34]. Less is known, however about the physiological role of centrally produced GLP-1, the mechanisms of its release, and the site of its action.

GLP-1R are expressed at both peripheral and central sites [2, 6, 35, 36, 36, 37]. The importance of centrally expressed GLP-1R in BW regulation is widely accepted [9, 18, 38]. Because many investigators use long-acting

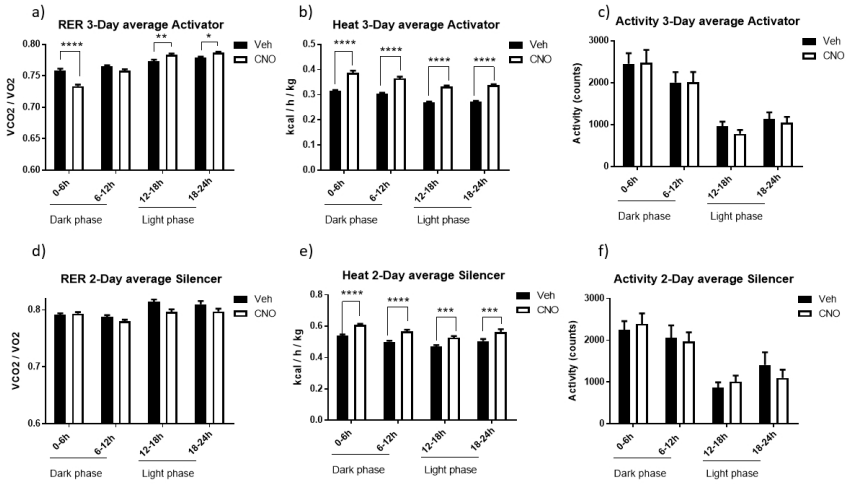

FIGURE 2.7: Energy expenditure after DREADD-mediated PPG neuronal activation or silencing in DIO mice: a) RER after chronic activation; $n = 6$, two-way ANOVA $p < 0.0001$, with treatment effect $*p < 0.05$, $**p < 0.005$, $****p < 0.0001$ b) heat after chronic activation; $n = 6$, two-way ANOVA $p < 0.0001$, with treatment effect $****p < 0.0001$, c) activity after chronic activation; $n = 6$, two-way ANOVA; no significance, d)RER after chronic silencing, $n = 4$, two-way ANOVA, no significance, e) heat after chronic silencing; $n = 4$, two-way ANOVA $p < 0.0001$, with treatment effect $****p < 0.0001$, f) activity after chronic silencing; $n = 4$ two-way ANOVA, no significance.

GLP-1R agonists to study the physiological role of GLP-1, it is difficult to judge on the physiological relevance of the findings in terms of endogenous GLP-1 functions. Because gut-derived GLP-1 has a short half-life (less than 2 min in the blood) [6, 34, 39–41], it is unlikely that peripheral GLP-1 crosses the blood brain barrier (BBB) and acts directly on GLP-1 receptors in the brain. Importantly, a recent study from our laboratory demonstrated that the full satiating effect of peripheral endogenous GLP-1 requires GLP-1R on vagal afferent neurons, presumably terminating close to the site of GLP-1 release [33]. Previous studies have shown that centrally administered GLP-1R agonists inhibit food and water intake and that these effects require functional GLP-1R in the ARC [9, 42]. Because a direct activation of central GLP-1R by peripherally produced GLP-1 is unlikely, centrally produced GLP-1 may play a major role in regulating the forebrain neural circuits important for FI and BW regulation [38].

Previous studies have reported that PPG mRNA expression in the NTS is up-regulated in DIO mice [10]. These studies demonstrated that a central GLP-1 action during HFD feeding resulted in hyperinsulinemia and insulin resistance and that blocking this action with ICV administration of the GLP-1R antagonist exendin-9 prevented the HFD-induced diabetic phenotype [10]. It is therefore important to understand the role of GLP-1 producing neurons in the pathophysiology of DIO. Here, we characterized the time course of central PPG mRNA upregulation during HFD feeding and the development of obesity. We demonstrate that reducing PPG expression using viral-mediated RNA interference ameliorates hyperphagia and slows BW gain in HFD-induced obese mice. Furthermore, we investigated the role of leptin signalling in PPG gene regulation and provide evidence that adiposity, whether it is due to lack of leptin or linked to high leptin levels, is associated with PPG mRNA overexpression. Lastly, using the chemogenetic DREADD approach, we demonstrated that a pharmacological activation of PPG neurons reduces FI and BW in DIO mice, identifying PPG neurons as a potential target for the treatment of obesity.

We found that PPG mRNA expression increases during the course of HFD feeding and DIO development. Moreover, we showed that the number of PPG expressing neurons in the NTS was higher in DIO mice compared to that of mice fed CD. This increase in PPG neuron numbers may also explain the increase in PPG mRNA levels. It is however unknown which neural or hormonal factor stimulates PPG gene expression, and which neurons become PPG expressing neurons after HFD exposure. That PPG mRNA overexpression is associated with increased BW and plasma

leptin levels during HFD feeding prompted us to hypothesize that leptin might be the major driver of PPG upregulation. We found PPG mRNA up-regulation in obese diabetic ob/ob and db/db mice, which was reduced by peripheral leptin administration in ob/ob but not in db/db mice, suggesting that leptin can modulate PPG gene expression. A previous study reported that fasting, a state of low circulating leptin, decreases hindbrain PPG mRNA expression, and exogenous leptin treatment fully reversed this phenotype in normal chow-fed mice. In our study, peripheral leptin injection failed to increase PPG levels in WT littermate controls of ob/ob or db/db mice. This discrepancy between our data and published findings could be due to the difference in fasting duration, i.e., 2 h (our study) vs. 24 h fasting (Huo et al. [11]). Whereas a two-hour food deprivation hardly affects circulating leptin levels, prolonged fasting significantly decreases them, which is a starvation signal sensed by the brain. Brain PPG mRNA expression is also reduced after prolonged fasting, and leptin administration can restore PPG levels [11], indicating that PPG expression is an important hindbrain marker for feeding status. Although we did not measure circulating leptin levels, it is unlikely that 2 h food deprivation caused a significant drop in leptin. In other words, our data suggest that, in the presence of endogenous leptin, additional leptin administration has little influence on PPG levels. This is reminiscent of the fact that leptin administration strongly affects FI and EE when endogenous leptin levels are low, but that very high, pharmacological leptin doses are usually necessary to affect these endpoints when leptin levels are normal or high [43].

Our data also suggest that hyperleptinemia induced by overweight is closely associated with PPG overexpression. Because leptin regulates PPG neurons via functional LepRs, we hypothesized that hyperleptinemia [44] is the major driver of PPG mRNA up-regulation in obesity. Yet, contrary to our assumption, PPG mRNA overexpression was partially reversed by leptin injection in ob/ob mice, but not db/db mice, indicating that PPG gene regulation by leptin indeed requires functional LepRs, and that leptin can normalize PPG overexpression in a leptin-deficient state. Because both ob/ob (leptin deficiency) and DIO mice (hyperleptinemia) express high levels of PPG, it appears that PPG expression increases with obesity and hyperphagia. Intriguingly, leptin injection resulted in a decrease of PPG levels in ob /ob mice, raising the possibility that either too little or too much leptin contributes to PPG overexpression. In other words, whether exogenous leptin decreases or increases PPG expression may depend on the energetic state of the organism. In negative energy balance, such as

during fasting, exogenous leptin may restore PPG expression. On the other hand, in case of leptin deficiency that leads to a positive energy balance, exogenous leptin rapidly corrects the PPG overexpression [11], which may be one of the mechanisms contributing to the FI reduction by leptin under these conditions. Remarkably, we showed that HFD feeding increases not only PPG mRNA levels but also the number of PPG expressing neurons in the NTS compared to chow-fed animals. The increase in PPG neurons may explain - or at least contribute to - the increased PPG mRNA levels. Additional questions however remain unanswered, including which signals drive this change in the neurochemical phenotype and which neurons become new PPG neurons after the exposure to HFD.

We found that mice kept on CD expressed P-STAT3 in PPG neurons after peripheral leptin administration. Previous studies have also shown that leptin depolarizes PPG neurons derived from chow-fed mice, demonstrating that they are leptin sensitive [20]. ARC neurons become resistant to leptin action with HFD-feeding, but other neurons in the hypothalamus remain responsive to leptin. It is unclear whether hindbrain PPG neurons develop leptin resistance or still respond to leptin after long-term HFD exposure. To our knowledge, there is no evidence in the literature showing altered leptin sensitivity in hindbrain neurons after HFD-feeding. However, it has been shown that deletion of the LepR in the hindbrain using Phox2b Cre LepRflox/flox mice makes these animals hyperphagic and gain weight compared to WT controls [19]. To test whether hyperleptinemia/leptin resistance contributes to hindbrain PPG overexpression and hyperphagia in DIO mice, we blocked leptin receptor signalling in the hindbrain by injecting leptin receptor antagonist (LRA) into the 4^{th} ventricle and measured the effects on BW and FI. We hypothesized that reducing leptin signalling in the hindbrain improves energy balance by normalizing PPG mRNA levels, and thereby decreasing FI and BW. In fact, we found that 4^{th} ventricle infusion of the LRA reduced FI, BW, and tended to decrease PPG mRNA levels. Our data therefore imply that leptin signalling contributes to the hyperphagia that is linked to PPG overexpression in the hindbrain. Because the PPG gene produces GLP-1 as an anorexigenic signal, PPG overproduction could increase GLP-1 projections to the forebrain where GLP-1R are highly expressed. DIO animals are however hyperphagic despite the PPG overproduction, which suggests that GLP-1R signalling in the forebrain leads to abnormal eating behavior in obesity. It is however unclear whether blocking LepR signalling reduces FI by directly affecting PPG production or indirectly via other LepR expressing neurons locally connected to PPG

neurons. Considering the therapeutic value of central GLP-1 function, it is important to better understand how leptin affects GLP-1R signalling in DIO.

Long-term HFD feeding increased BW as well as NTS PPG mRNA expression compared to chow feeding, suggesting that the central PPG expression level, and possibly the GLP-1 level, is positively correlated with obesity. To test whether reduced central PPG production alleviates DIO phenotypes, we used lentiviral-mediated RNA interference (LV-shPPG) to knockdown (KD) NTS PPG mRNA expression. PPG shRNA injection into the NTS decreased PPG expression, BW gain and daily FI in adult male mice fed HFD for over 18 weeks. Our data suggest that increased PPG levels indicate a positive energy balance and that reducing this expression directly affects eating behaviour and BW. In line with the decreased FI, hypothalamic NPY expression was significantly lower in KD mice compared to control mice, whereas POMC, AgRP and CART mRNA expression remained unchanged. This decrease in hypothalamic NPY levels in response to a reduction of hindbrain PPG expression could be one of the mechanisms through which PPG neurons inhibit FI in this situation. The exact mechanism of PPG-derived peptide action on NPY neurons is not well understood, and in our study we were unable to identify which hypothalamic NPY neurons were affected by our manipulation because we took the entire hypothalamus for our gene expression analysis. Previous viral tracing studies however have shown that NTS neurons have projections to NPY neurons in the dorsomedial hypothalamus (DMH), raising the possibility that PPG neurons could be among those neurons directly projecting to DMH NPY neurons that contribute to hyperphagia in DIO.

A recent study showed that PPG neurons play a role in glucose metabolism and insulin sensitivity [45]. Acute activation of PPG neurons in lean animals enhanced glucose tolerance, decreased basal endogenous glucose production and increased insulin sensitivity [45]. In our study an oral glucose tolerance test did however not reveal any difference in glucose sensitivity between KD and control mice. The different outcome of the two studies could be due to the difference in the animals' nutritional and metabolic states: In our study the mice were obese due to the HFD exposure, and were then challenged with the OGTT, whereas the authors of the previously published study [45] used lean mice. Presumably, the function of PPG neurons greatly differs depending on the feeding status.

We also assessed the effects of PPG neuronal activation or silencing on FI and BW to compare the outcome with the PPG gene knockdown. Pre-

viously, PPG neuronal activation using DREADD technology in mice decreased FI [19]. The caveat with respect to the previous study is that the authors used Phox2B Cre mice [19], which is not specific for PPG neurons. We confirmed that acute modulation of PPG neurons using a PPG neuronal specific mouse model decreased (activation) and increased (silencing) FI in both chow and HFD-fed mice. In addition, we also characterized the consequences of chronic PPG neuronal modulation on FI and EE in DIO mice. Overall PPG neuronal activation decreased short-term FI and RER, but increased EE in DIO mice. This increase in EE appears to be largely responsible for the BW loss because overall FI was unchanged after chronic PPG neuronal activation. Paradoxically, we show that both PPG KD and PPG neuronal activation lead to a decrease in FI. Interestingly, PPG neuronal activity did not alter the PPG expression level, suggesting the mechanisms that lead to an inhibition of eating differ between the two models.

The anorexigenic effect of central GLP-1 is partially mediated by the mesolimbic dopaminergic circuit involved in reward and motivation [19, 46]. Moreover, GLP-1 neurons have been implicated in mediating the stress response and visceral malaise [46]. This is based on studies showing that GLP-1 activates the HPA axis as well as the sympathetic nervous system [46]. PPG neuronal activation may therefore lead to an activation of the stress response and thereby affect FI and EE. Glutamatergic neurons in the NTS control the excitation of the parabrachial nucleus (PBN) [47], an important nucleus in the brainstem integrating signals from different brain regions with the capacity to modulate FI and BW [47,48]. Direct delivery of a GABA receptor partial agonist into the PBN was shown to be sufficient to maintain feeding, and inactivation of GABA receptors was shown to lead to anorexia. Moreover, loss of GABA signalling as a result of AgRP neuronal lesion disinhibits PBN neurons, and consequently inhibits FI [48]. Blocking glutamatergic signalling from the NTS prevents this starvation response and maintains normal eating after AgRP neuronal lesion [47,48]. Because NTS PPG neurons are also glutamatergic [49], it is possible that the activation of the NTS-PBN pathway is involved in the reduction in FI in response to DREADD-mediated PPG neuronal activation. Either way, our data suggest that PPG neuronal activation may be a viable therapeutic strategy for the treatment of obesity.

Overall, our results suggest that elevated central GLP-1 levels contribute to the DIO phenotype by modulating hypothalamic neural circuits involved in the control of FI. This also indicates that PPG neuronal activation and PPG mRNA upregulation doesn't produce the same results. Previous stud-

ies in rodents and humans have extensively focused on beneficial effects of peripheral administration of GLP-1 analogues and their antiobesity and insulinotropic actions in patients with T2DM [5,6,15,16,34,41,50,51]. Because peripheral GLP-1 secretion is compromised in obesity, peripheral administration of long lasting GLP-1R agonists is one strategy to improve GLP-1 actions in the periphery as well as the brain [9,17,42,51–54]. However, central GLP-1 signalling appears to be involved in a wider range of functions beyond FI and blood glucose regulation [1,6,9,18,21,34,37,38,45,46]. Therefore, understanding the physiological functions of PPG neurons in normal and pathological states is of great interest to improve GLP-1-based therapies. In sum, our findings provide new evidence that GLP-1 neurons and signalling mechanism could be a potential target for drug development toward combating obesity.

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3

INTESTINAL LYMPH AS A READOUT OF MEAL–INDUCED GLP-1 RELEASE

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3.1 ABSTRACT

Intestinal lymph supposedly provides a readout for the secretion of intestinal peptides. We here assessed how mesenteric lymph duct (MLD) lymph levels of glucagon-like peptide (GLP-1), insulin, and metabolites (Glucose, triglycerides [TG]) evolve after isocaloric high and low fat diet (HFD, LFD) meals, and how they compare to hepatic portal vein (HPV) plasma levels. Moreover, we examined the effects of intraperitoneally (IP) administered GLP-1 (1 or 10 nmol/kg) on these parameters. At 20 min after the HFD meal onset, GLP-1 levels were higher in MLD lymph than in HPV plasma. No such difference occurred with the LFD meal. IP injections of 10 nmol/kg GLP-1 prior to meals enhanced the meal-induced increases in MLD lymph and HPV plasma GLP-1 levels except for the MLD lymph levels after the HFD meal. IP injection of 1 nmol/kg GLP-1 only increased HPV plasma GLP-1 levels at 60 min after the HFD meal. GLP-1 injections did not increase the MLD lymph or HPV plasma GLP-1 concentrations beyond the physiological range, suggesting that IP GLP-1 injections can recapitulate short-term effects of endogenous GLP-1. DPP-IV activity in MLD lymph was lower than in HPV plasma, which presumably contributed to the higher levels of GLP-1 in lymph than in plasma. Insulin and glucose showed similar profiles in MLD lymph and HPV plasma, whereas TG levels were higher in lymph than in plasma. These results indicate that in-

testinal lymph provides a sensitive readout of intestinal peptide release and potential action, in particular when fat-rich diets are consumed.

3.2 INTRODUCTION

Interstitial fluid surrounds cells, providing them with nutrients and participating in waste removal. Interstitial fluid composition in a tissue therefore reflects the local cellular microenvironment. Lymph provides the best readout of interstitial fluid because it drains the interstitial fluid and its contents from the tissues, including compounds that do not reenter the blood stream [6, 15]. Intestinal lymph transports the bulk of absorbed lipids as chylomicrons [8, 27, 34, 36], and it contains high concentrations of gastrointestinal (GI) peptides, which enteroendocrine cells secrete into the interstitial fluid in relation to eating [15, 20]. Lymph composition differs from blood or plasma composition due to differences in the fluid and substrate exchange processes [6, 11]. Also, the concentrations and activities of various enzymes differ between lymph and plasma [7, 38]. The analysis of intestinal lymph is therefore interesting with respect to several questions related to fat handling and the physiological role of GI peptides. In addition, there is evidence for disproportional transport of GI peptides by lymph compared to blood [7, 38]. Mainly because of technical difficulties of the sampling, however, lymph has not been studied as extensively as blood or blood plasma.

Most previous studies addressing lymph composition used animals that were restrained, for instance in the Bollman cage, in which animals presumably experience massive stress. In this setup the intestinal lymph is continuously drained, and animals are sacrificed within 48 hours of experiment onset [5]. Some other studies used anesthetized instead of restrained animals, but in both cases the animals could not normally behave or eat, hence, none of these experiments reflected physiological conditions [7, 11, 25]. We previously described a novel technique of chronic superior mesenteric lymph duct (MLD) cannulation in rats [2]. This technique allows for repetitive sampling of intestinal lymph in relation to spontaneous real meals in undisturbed, freely behaving rats. The MLD is one of three lymph ducts that drain the abdominal viscera [34]. We chose it because it is 1) the major lymph duct draining the small intestine [35] and 2) big and easy to identify next to the superior mesenteric artery [19].

Here we further refined this technique and used it in rats together with a chronic hepatic portal vein (HPV) cannulation to 1) further investigate the MLD lymph GLP-1 concentrations in response to isocaloric purified high fat diet (HFD) and low fat diet (LFD) meals and 2) to compare them to HPV plasma concentrations of GLP-1. Moreover, 3) we measured the MLD lymph and HPV plasma concentrations of GLP-1 after IP injections of GLP-1 at a dose that reliably reduces food intake in rats [31,33,39]. We did this to judge whether such injections produce physiologic or pharmacologic concentrations of GLP-1 at the presumed site of its action, i.e., near the vagal afferent terminals in the wall of the small intestine. 4) We also measured the activity of the GLP-1 degrading enzyme DPP-IV in MLD lymph and HPV plasma in response to the HFD or LFD test meals and in response to IP GLP-1 injection (10 nmol/kg). There is evidence that even short-term HFD exposure can attenuate the eating-inhibitory effect of IP administered GLP-1. Comparing MLD and HPV GLP-1 levels in response to GLP-1 injections in rats given a HFD or LFD test meal may reveal whether lower concentrations of GLP-1 at the putative site of action contribute to the attenuation of the satiating effect of exogenous GLP-1 by short-term HFD exposure. Finally, 5) we measured the concentrations of insulin as well as some fat and carbohydrate metabolites in MLD lymph and HPV plasma to estimate whether there are major differences in addition to the anticipated differences in triyglyceride (TG) levels.

3.3 MATERIALS AND METHODS

Animals and surgery: The Cantonal Veterinary Office of Zurich approved all experimental procedures. Male SD rats were purchased from Charles River (Sulzfeld, Germany) at an average BW of $250 - 300$ g. The rats were maintained on a 12 : 12 h light-dark cycle, with lights on at 12 : 00 am, and had free access to standard laboratory chow and water unless noted otherwise. Prior to surgery, animals were fasted overnight starting from 10 pm, i.e., towards the end of their light phase. One hour before surgery, animals were subcutaneously (SC) injected (1 ml/kg body weight) with a mixture of 20 mg/kg Sulfadoxin and 5 mg/kg Trimethoprim (Borgal 24%, Intervet, Shering-Plough Animal Health, Kenilworth, NJ). Then the animals were gavaged with 1.5 ml olive oil $1 - 2$ h prior to anesthesia. The oil gavage served to increase lymph flow in the mesenteric lymph duct (MLD) and gave the otherwise almost transparent lymph duct a whitish-opaque color, which facilitated identification of the catheter insertion place.

Fifteen to 30 min prior to anesthesia 0.05 mg/kg atropin (Sintetica, Mendrisio, Switzerland) was injected SC (1 ml/kg). An IP injected mixture of xylazine (5 mg/kg, Rompun; Bayer, Leverkusen, Germany) and ketamine (86 mg/kg, Ketasol-100; Dr. E. Gräub, Bern, Switzerland) was used for anesthesia.

Three catheters were implanted during the surgical procedure: an intraperitoneal (IP) catheter, a hepatic portal vein (HPV) catheter and a MLD catheter. All catheters were prepared as described before [2]. Briefly, the IP catheter was assembled using 17 cm silicone tubing (ID 0.508 mm, OD 0.914 mm; Gore WL, Newark, DE), with a 22 G V-shaped cannula polished at both ends attached to the proximal end, and with 12 holes poked in its other end using a 26 G cannula. The dead space of an IP catheter was 40 mL. The HPV catheter was assembled using 23 cm polyurethane (PU) tubing (Art. MRE-040 ID 0.60 mm, OD 0.96 mm Microrenathane, Braintree Scientific, Braintree, MA, USA.). The PU tubing had a 22 G cannula attached to one end, and its other end was tapered to about 0.7 mm by pulling it in 160 °C mineral oil. The dead space of an HPV catheter was 80 µL. The MLD catheter was assembled using 22 cm PU tubing (Art. MRE-025; ID 0.3mm OD 0.64 mm; Microrenathane, Braintree Scientific, Braintree, MA). The PU tubing had a 26 G V-shaped cannula attached on one end, and the other end was tapered to about 0.15 mm OD by pulling in 160 °C mineral oil. The dead space of the MLD catheter was about 15 µL. The proximal ends of all three catheters were sutured to polypropylene surgical mesh (Marlex; Bard Implants, Billerica). Before implantation all catheters were sterilized in disinfectant solution (Kodan-Tinktur forte farblos; Schluer and Mayr, Norderstedt, Germany) and rinsed with 0.9% NaCl. Surgery was performed under aseptic conditions using a surgical microscope.

After implanting the polypropylene mesh with the infusion ports subcutaneously at the between the scapulae the catheters were guided subcutaneously to a 4 cm ventral skin incision. After laparotomy, the tubings were inserted into the abdominal cavity through puncture holes in the abdominal muscle and a drop of medical glue (Histoacryl®, B. Braun, Melsungen, Germany) was applied to the insertion area of the silicon tubing into the abdomen, to fix them in place. The HPV catheter implantation followed a previously described procedure [4] with some modifications. Briefly, the catheter was inserted into the superior mesenteric vein at the level of the splenic vein and advanced around 6 mm to place its tip about 1 mm down-

stream of the gastroduodenal vein, where it ended freely in the HPV. This way the disturbances of intestinal blood flow are minimal [32]. After inserting the catheter, the tubing was fixed in its place using 4-0 silk sutures. The MLD catheter was implanted as previously described [2] and was stabilized in the lymph duct using $5-6$ mm Vialon® tubing (0.9×25 mm 381223 or 0.7×19 mm 381212, BD Insyte IV Catheters; Becton Dickinson, Madrid, Spain), which was placed in the duct first. The Vialon® tubing had a 0.15 mm hole in its wall allowing for the insertion of the MLD catheter.

After surgery, approximately 3 ml of warm 0.9% NaCl and Ringers lactate solution were injected through the IP catheter. For 3 days after surgery Carprofen (5 mg/kg, Norocarp, UFAMED AG, Sursee, Switzerland) was given SC. Borgal was prepared as described above and injected SC on the first post-surgery day. The animals were closely monitored after surgery. On average they lost less than 4% BW through the surgery and were steadily gaining weight thereafter, reaching 99.8% of pre-surgery BW within 7 days post-surgery, when the blood and lymph sampling started. Moreover, during the recovery, animals did not show any visual sign of suffering; they were always alert and well-groomed.

3.3.1 *Catheter maintenance*

IP catheters were always filled with 0.9% NaCl solution (B. Braun), whereas for the HPV and MLD catheters a lock solution of 50% glycerol containing 200 IU heparin/mL was used. All catheters were flushed daily. On experimental days, the glycerol solution of the HPV an MLD catheter was removed by aspiration, both catheters were well rinsed and filled with NaCl solution 30 min prior to the baseline measurement.

Also the lymph catheter filling solution was aspirated before each sampling. To do so, a saline-filled tubing, connected to a syringe, with a small air bubble at the distal end, was connected to the infusion port. Then the catheter filling was slowly aspirated in a way that the air bubble moved less than 20 cm/min, corresponding to no more than 15 µL pec minute. Although the lymph in the tube (10 µL) changed colour quickly, we aspirated and discarded two times the dead space of the catheters (i.e., 20 µL), before the sampling reservoirs were attached and the lymph samples collected, in order to avoid contamination of the lymph with rinsing fluid.

After completion of the lymph sampling, the catheters were flushed with physiological saline by infusing one time the volume of the dead space within 1 min. This way we just filled the catheters and did not infuse substantial amounts of rinsing solution into the lymph duct. After the samplings were completed, catheters were flushed and filled with heparinized glycerol (one volume of dead space).

3.3.2 *Lymph and blood sampling in relation to test meal with or without IP GLP-1 injection*

The three catheters described above allowed for parallel sampling of MLD lymph and HPV blood as well as for peptide administration. Experimental sampling started one week after surgery, when rats had recovered and regained their pre-operative BW. During the recovery period regular chow (Provimi Kliba AG, Kaiseraugst, Switzerland #3436) was used.

On experimental days, 1 h into the dark phase, baseline samples of lymph and blood were taken and rats were injected through the IP catheter with 10 mM PBS containing 0 (vehicle), 1, or 10 nmol/kg GLP-1 and 1% BSA in a total injection volume of 1 ml. Immediately after the injections, rats were offered isocaloric high fat diet (HFD, 1.75 g, 60 kJ% fat, SSNIFF E15742 – 34 EF D12492 (I) mod) or low fat diet (LFD, 2.3 g, 10 kJ% fat, SSNIFF S9213-E001 EF D12450B (I) mod) test meals (SSNIFF Spezialdiäten GmbH, Soest, Germany). To achieve equal calorie contents of the test meal, we gave different amounts of food, i.e., 1.75 g of the calorically more dense HFD or 2.30 g of the calorically less dense LFD. The rats had been trained to complete the test meals within 3-6 min three times between surgery and the experiment. Only rats that completed their test meal within 2-3 min after injection underwent parallel lymph and blood samplings at 20, 40, 60, 120 and 180 min after injection.

Lymph (120 µL) and blood (250 µL) samples were cooled during collection and treated with 540 KIU/ml Aprotinin (Sigma-Aldrich, Buchs Switzerland # A6106) and 10 µLml DPP-IV inhibitor (200 µLml, catalog no. DPP4, Millipore, Billerica, MA). Lymph samples were frozen in liquid nitrogen immediately after collection. All blood samples were centrifuged at 9.500 x g for 10 min, and supernatant serum after clotting and plasma when anticoagulants were used was stored at −80°C until further analysis. These samples were used for all hormone and metabolite analyses.

3.3.3 *GLP-1 and insulin measurements*

Active GLP-1 and insulin in MLD lymph and HPV plasma were analyzed using commercially available kits according to the manufacturer's specifications (Mesoscale Discovery, Rockville, MD, USA). GLP-1 using duplex (MSD, System Mouse/Rat total PYY/active GLP-1 K150JWC) and insulin using single spot (MSD, System Mouse/Rat Insulin Kit K152BZC) 96 MSD plates. The analyses were verified for linearity and checked for nonspecific immunoreactivity in MSD plates. The assay has already been extensively tested by the manufacturer for the use in EDTA rat plasma. As reported, spiking of plasma with various amounts of the calibrators to produce levels throughout the range of the assay yielded a recovery between 79 and 85%. Linearity as measured by spiking of pooled EDTA rat plasma samples with calibrator, followed by subsequent dilution, yielded a recovery rate between $98 - 120\%$. To validate the assay for lymph samples we ran dilution series of spiked pooled lymph and found acceptable linearity from $1 : 2$ to $1 : 10$ ($79 - 133\%$, mean 101%). Recovery was determined by spiking of 10 individual lymph samples ranging from $14 - 120$ pg/mL, yielding a recovery of $79 - 100\%$, with an average of 88%.

3.3.4 *Glucose and TG measurements*

Commercially available kits (Glucose, Triglyceride Hoffman La Roche, Switzerland, and FFS WAKO Chemicals GmbH, Neuss, Germany) adapted to the Cobas Mira autoanalyzer (Hoffman La Roche, Switzerland) were used for the analyses of glucose and TG. Samples were diluted 12 fold for TG measurement and 5 fold for the measurement of glucose; all samples were briefly centrifuged before analysis.

3.3.5 *DPP-IV activity assay*

Separate animals were used for the DPP-IV activity assay. The same procedure was used and the same volumes of lymph and blood were collected as for the hormone and metabolite analyses, but no additives or inhibitors were added, and blood was allowed to clot for 20 min at room temperature. The commercially available Activity assay kit (Sigma Aldrich, MO) was used to measure DPP-IV activity. Briefly, this assay uses a non-fluorescent substrate for cleavage of H-Gly-Pro linked to Fluorescent Amino-4-Methyl Cumarin (AMC). AMC is released upon cleavage of a non-fluorescent sub-

strate, and the activity of one unit corresponds to the amount of enzyme that is required to hydrolyze the substrate to 1 mmol yield of AMC per minute at 37°C. Final DPP-IV activity is reported as $pmol/min/mL = microunits/mL$.

3.3.6 *Data analysis*

The data are presented as means ± standard error of the mean (S.E.M.). The statistical analysis was performed using the SAS program (Version 4.3). The evolution of hormones and metabolites over time was analyzed for significant differences ($p < 0.05$) using a three-way ANOVA (diet x drug x time) with Bonferroni post-hoc tests. The graphs were generated using GraphPad Prism software (Version 7).

3.4 RESULTS

3.4.1 *The HFD and LFD test meals increased MLD lymph and HPV plasma GLP-1 concentrations*

The HFD test meal increased endogenous GLP-1 concentrations compared to baseline only at 20 min after meal onset ($p < 0.05$ vs. baseline) in MLD lymph, but not in HPV plasma Fig. 3.1a. At later time points after the HFD test meal, the GLP-1 concentration in MLD lymph appeared to remain elevated, but the difference to baseline values was not statistically significant. The LFD test meal tended to transiently increase endogenous GLP-1 levels in MLD lymph around 40 min Fig. 3.1b, but the difference to baseline was also not significant. The LFD test meal did transiently increase GLP-1 levels at 20 min in HPV plasma ($p < 0.05$ vs. baseline). The AUC analysis (0-180 min with baseline as a reference) for endogenous GLP-1 shows that the HFD test meal increased ($p < 0.01$) GLP-1 in MLD lymph more than in HPV plasma, whereas the LFD test meal increased GLP-1 in MLD lymph and HPV plasma similarly Fig. 3.1c.

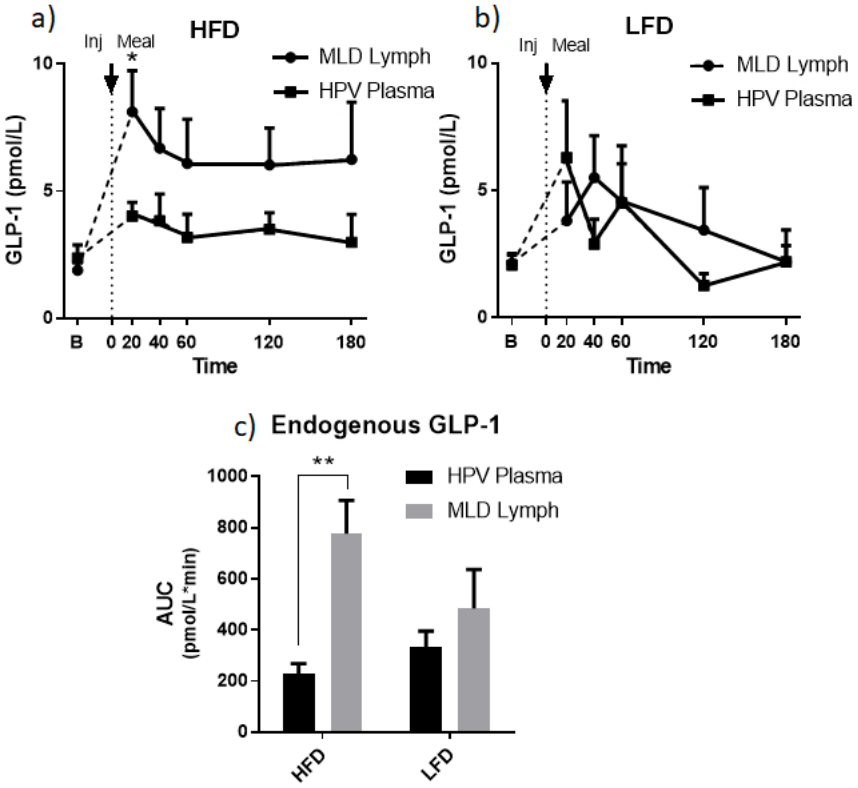

FIGURE 3.1: Endogenous release of GLP-1 in response to isocaloric HFD or LFD test meals. Baseline was sampled approximately 20 min prior to vehicle injection and a test meal. Vehicle was injected at time zero, and the HFD or LFD test meal was offered immediately thereafter. GLP-1 in HPV blood plasma (a) and in MLD lymph (b). Area under the curve (AUC, 0 to 180 min above the baseline) for endogenous GLP-1 (c). Data represent means ± S.E.M with $n = 5/6$. The evolution of GLP-1 levels over time was analyzed for significant differences ($p < 0.05$) using a two-way ANOVA (diet x time) with Bonferroni post-hoc tests. For clarity, only key significant differences are indicated. *higher ($p < 0.05$) than baseline MLD lymph value. **greater ($p < 0.01$) than corresponding HPV plasma AUC value.

3.4.2 *IP GLP-1 injections increased MLD lymph and HPV plasma GLP-1 concentrations independent of test meal fat content*

IP injection of 10 nmol/kg GLP-1 increased ($p < 0.05$) GLP-1 concentration in HPV plasma and MLD lymph at 20 and 40 min compared to vehicle with the HFD and LFD test meals Fig. 3.2. With the HFD test meal, IP injection of 1 nmol/kg GLP-1 tended to increase the GLP-1 concentration in HPV plasma and MLD lymph, but the differences compared to the endogenous release (vehicle) were not significant. With the HFD test meal, the IP 1 nmol/kg GLP-1 dose produced an almost significant increase in GLP-1 levels compared to baseline in MLD lymph at 20 ($p = 0.057$) and 40 min ($p = 0.06$) post injection Fig. 3.2b. Compared to the 10 nmol/kg GLP-1 dose, the highest GLP-1 level after IP injection of 1 nmol/kg GLP-1 appeared to be reached later (at 40-60 rather than 20 min) with the HFD test meal in HPV plasma and with the LFD test meal in MLD lymph. As a result, with the LFD test meal, the GLP-1 level in MLD lymph was lower ($p < 0.05$) after IP injection of 1 nmol/kg GLP-1 than after injection of 10 nmol/kg GLP-1. There was no such difference with HFD test meal.

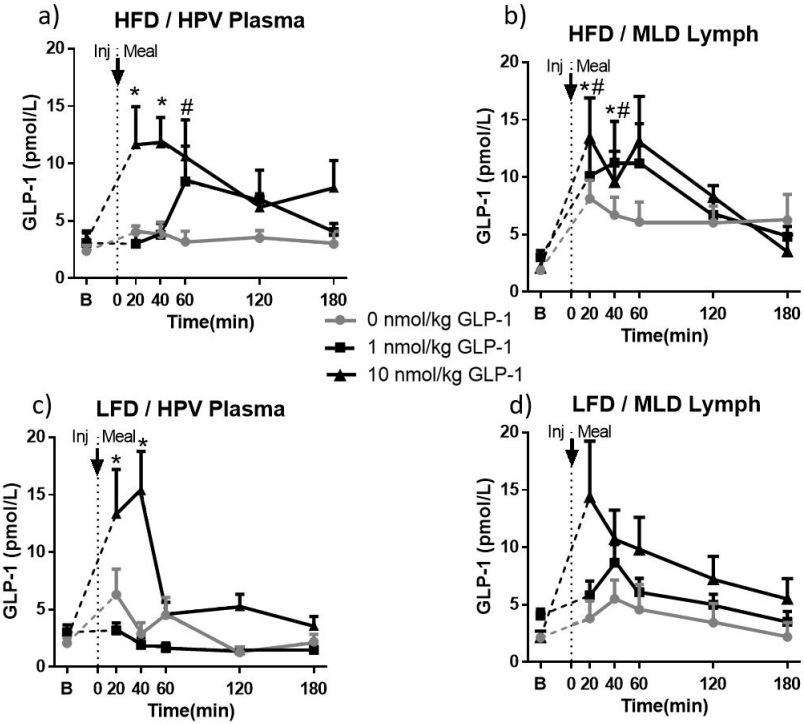

FIGURE 3.2: Levels of GLP-1 in MLD lymph and HPV plasma in response to IP injections of 0 (vehicle) 1 or 10 nmol/kg GLP-1 immediately prior to isocaloric HFD or LFD test meals. Baseline was sampled approximately 20 min prior to injection and test meal. Please note that the curve in light grey color represents the endogenous GLP-1 release upon vehicle injection that is depicted on Fig. 3.1, is added here for comparison. Data represent means ± S.E.M with $n = 5/6$. The evolution GLP-1 over time was analyzed for significant differences ($p < 0.05$) using a three-way ANOVA (diet x drug x time) with Bonferroni post-hoc tests. For clarity, only key significant differences are indicated. #higher ($p < 0.05$) than 1 nmol/kg GLP-1 baseline value. *higher ($p < 0.05$) than 10 nmol/kg GLP-1 baseline value.

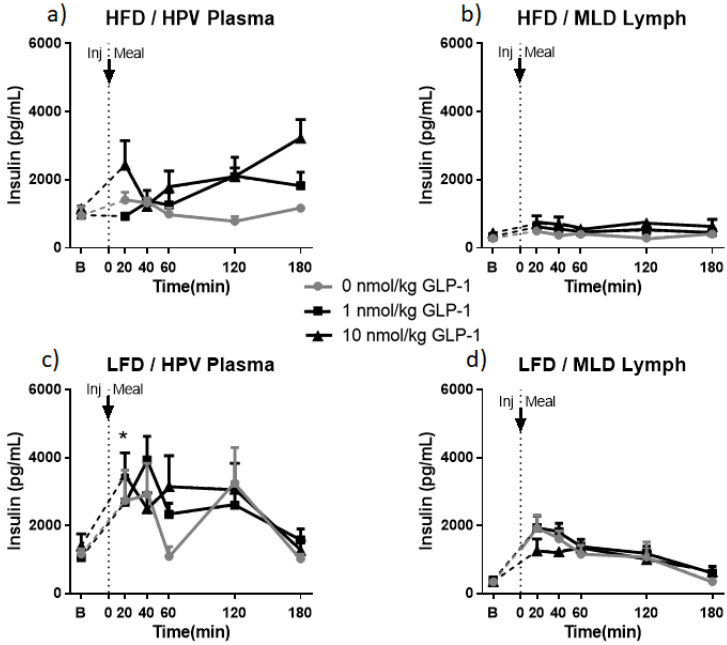

FIGURE 3.3: Levels of insulin in MLD lymph and HPV plasma in response to IP injections of 0 (vehicle) 1 or 10 nmol/kg GLP-1 immediately prior to isocaloric HFD or LFD test meals. Baseline was sampled approximately 20 min prior to injection and test meal. The curves in light grey color represent the endogenous insulin levels with vehicle injection. Data represent means ± S.E.M with $n = 5/6$. The evolution insulin over time was analyzed for significant differences ($p < 0.05$) using a three-way ANOVA (diet x drug x time) with Bonferroni post-hoc tests. For clarity, only key significant differences are indicated. *higher ($p < 0.05$) than 10 nmol/kg GLP-1 baseline value.

3.4.3 *Insulin, glucose and fat metabolites showed similar profiles in MLD lymph and HPV plasma*

The LFD, but not the HFD, test meal increased MLD lymph and HPV plasma insulin levels ($p < 0.05$ vs baseline). After the LFD test meal, the insulin concentration in MLD lymph reached its maximum at about 20 min, similar to the increase at 20 min in HPV plasma. IP GLP-1 injections (1 or 10 nmol/ kg BW) did not change the insulin levels compared to the endogenous release (vehicle), i.e., they did not change the insulin levels in response to the HFD test meal and did not increase insulin levels beyond the increase produced by the LFD test meal, neither in HPV plasma nor in MLD lymph. IP injection of 10 nmol/kg GLP-1 produced a trend towards an insulin increase 20 min post-injection, but this change did not reach statistical significance Fig. 3.3.

Independent from the test meals, and as expected, TG were present at much higher levels in MLD lymph compared to HPV plasma Fig. 3.4. With the LFD test meal, neither the test meal itself nor GLP-1 injections resulted in any significant changes in TG levels in HPV plasma Fig. 3.4. With the HFD test meal, however, the increase in TG levels was significant in HPV plasma at 120 and 180 min (both $p < 0.05$ vs baseline). Also, with the HFD test meal GLP-1 injection of 1 or 10 nmol/kg BW resulted in increased TG levels from 120 min post injection ($p < 0.0001$ and $p = 0.003$ vs baseline for 1 and 10 nmol/kg BW, respectively) to 180*min* post injection ($p < 0.001$ and $p < 0.0001$ vs baseline, respectively).

With the LFD test meal, the TG levels in MLD lymph tended to increase and almost reached significance at 60 min ($p = 0.06$ vs baseline). With GLP-1 injections (1 or 10 nmol/kg BW) TG levels increased 60 min post injection ($p < 0.01$ and $p < 0.0001$ compared to the baseline). After the HFD test meal, TG levels in MLD lymph increased ($p < 0.05$ vs baseline starting at 40 min). IP injection of 10nmol/kg GLP-1 prevented that increase. The LFD test meal increased the glucose levels in both HPV blood plasma and MLD lymph. These increases were unaffected by GLP-1 injections Fig. 3.5. With the HFD test meal neither the test meal nor GLP-1 injections had any detectable effect on HPV blood plasma or MLD lymph glucose levels at any time point measured.

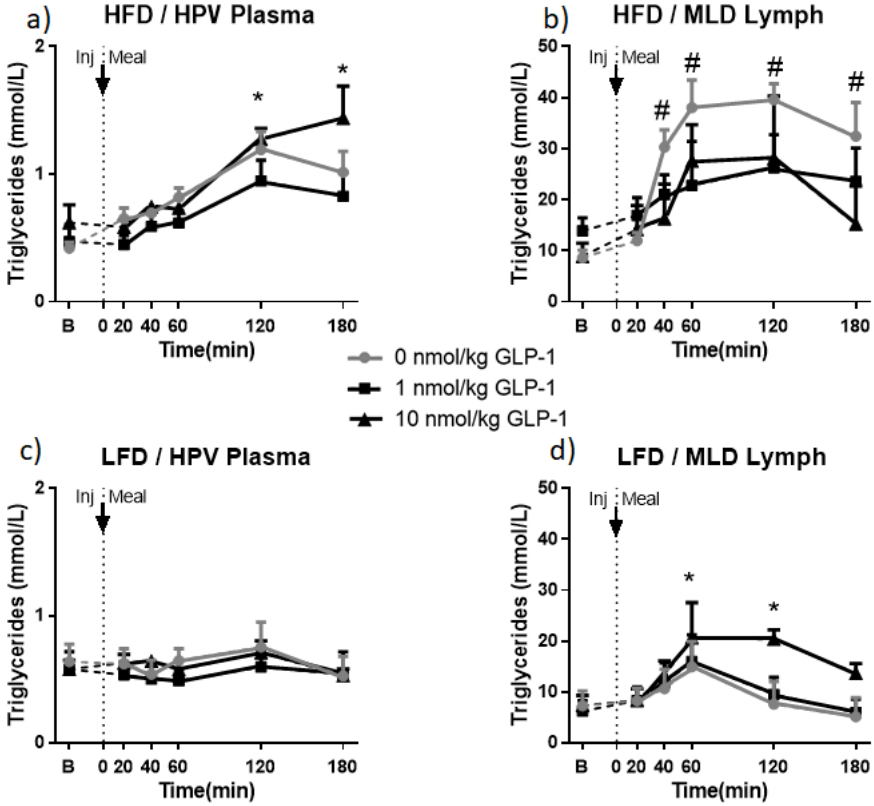

FIGURE 3.4: Levels of triglycerides in MLD lymph and HPV plasma in response to IP injections of 0 (vehicle) 1 or 10 nmol/kg GLP-1 immediately prior to isocaloric HFD or LFD test meals. Baseline was sampled approximately 20 min prior to injection and test meal. The curves in light grey color represent the triglyceride levels with vehicle injection. Data represent means ± S.E.M with $n = 5/6$. The evolution of triglycerides over time was analyzed for significant differences ($p < 0.05$) using a three-way ANOVA (diet x drug x time) with Bonferroni post-hoc tests. For clarity, only key significant differences are indicated. #higher ($p < 0.05$) than 0 nmol/kg GLP-1 baseline value. *higher ($p < 0.05$) than 10 nmol/kg GLP-1 baseline value.

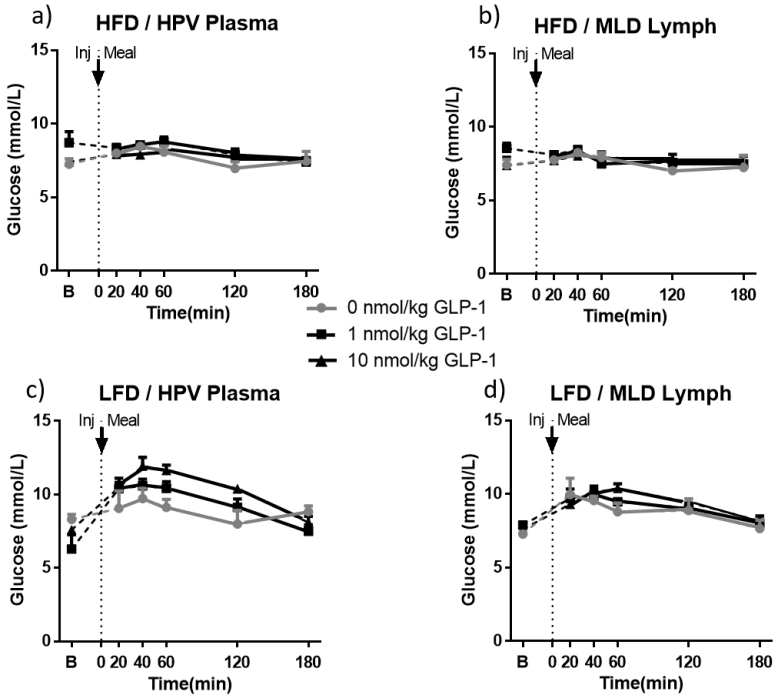

FIGURE 3.5: Levels of glucose in MLD lymph and HPV plasma in response to IP injections of 0 (vehicle) 1 or 10 nmol/kg GLP-1 immediately prior to isocaloric HFD or LFD test meals. Baseline was sampled approximately 20 min prior to injection and test meal. The curves in light grey color represent the glucose levels with vehicle injection. Data represent means ± S.E.M with $n = 5/6$. The evolution glucose over time was analyzed for significant differences ($p < 0.05$) using a three-way ANOVA (diet x drug x time) with Bonferroni post-hoc tests.

3.4.4 *DPP-IV activity was higher in HPV plasma compared to MLD lymph*

Overall levels of DPP-IV activity in HPV plasma were higher ($p < 0.05$ at all time points) compared to MLD lymph. The test meals themselves had no effect on DPP-IV activity Fig. 3.6. IP injection of 10 nmol/kg GLP-1 increased ($p < 0.05$) DPP-IV activity from 60 to 180 min post injection after the LFD test meal and from 40 to 180 min post injection after the HFD test meal. GLP-1 injection did not affect DPP-IV activity in MLD lymph.

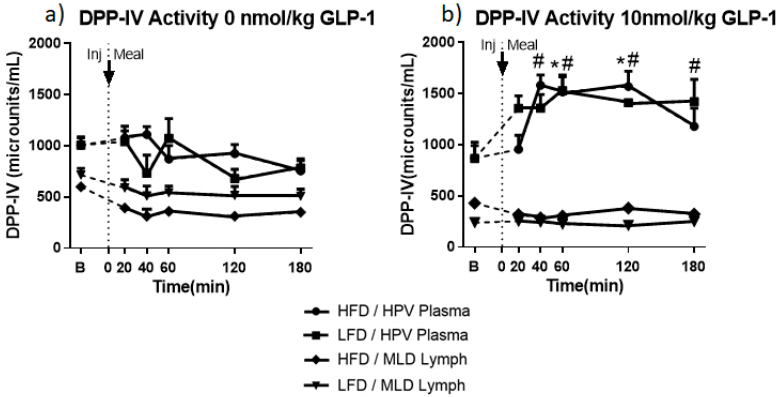

FIGURE 3.6: Levels of dipeptidyl peptidase IV (DPP-IV) Activity in MLD lymph and HPV plasma in response to IP injections of 0 (vehicle) or 10 nmol/kg GLP-1 immediately before isocaloric HFD or LFD test meals. Baseline was sampled $\sim$ 20 min before injection and test meal. Data representmeans ± S.E.M with $n = 6$. The evolution DPP-IV activity over time was analyzed for significant differences ($P < 0.05$) using a three-way ANOVA (diet compartment time) with Bonferroni post hoc tests. For clarity, only key significant differences are indicated. #Higher ($P < 0.05$) than HFD/HPV plasma baseline value. *Higher ($P < 0.05$) than LFD/HPV plasma baseline value.

3.5 DISCUSSION

The combination of our novel MLD catheterization technique with HPV catheters allowed for the first time to sample in parallel MLD lymph and HPV blood in relation to a test meal from unrestrained and un-anesthetized rats. In addition to the MLD and HPV catheters, the animals were equipped with IP catheters for easy administration of GLP-1. Compared to most previous studies investigating intestinal lymph, our unrestrained rat model provides several advantages: 1) The animals were not stressed during GLP-1 administrations or lymph/blood collection. 2) They could freely eat a test meal, which allowed for lymph and blood sampling in response to real meal taking. 3) The parallel sampling of MLD lymph and HPV blood allowed for a direct comparison of substance concentrations between the two compartments of the circulation. 4) Finally, we sampled the blood from the HPV, which is a more adequate sampling site to monitor the release of gut peptides such as GLP-1 than systemic blood, which most other studies used [11, 13, 23, 25]. With the HFD test meal, the GLP-1 measurements in MLD lymph detected an increase in GLP-1 release that was not identified in HPV plasma. This better detectability of a HFD meal-induced GLP-1 release in MLD lymph compared to HPV plasma is relevant to judge on physiological effects of GLP-1 under these conditions because intestinal lymph presumably provides the best readout of the interstitial fluid in the intestinal wall, which 'bathes' the vagal afferent nerve endings [9].

We assume that lymph truly represents the interstitial fluid around the intestinal endocrine cells because small intestinal lymphatics in the tissue collect the lymph from the interstitial fluid and form a series of collecting (afferent) lymphatic ducts, which transport lymph to the regional lymph nodes before it gets to the superior MLD, where we peformed the sampling. As lymph nodes are densely packed with lymphocytes, dendritic cells and macrophages, various molecules such as cytokines are also secreted into the lymph. Finally, some minor re-absorption of water and electrolytes, but not protein, occurs during the passage through the lymph nodes. To our knowledge, however, there is no evidence that any of our parameters of interest (concentrations of active GLP-1, glucose, triglycerides and DPP-IV activity) is affected significantly during the passage from the intestinal interstitial space to the collection site. Endogenous GLP-1 that is released in response to meals affects eating, gastric emptying, and glycemia at least in part by acting on GLP-1 receptors on these vagal af-

ferents terminating in the intestinal wall [17]. The HFD meal presumably increased MLD lymph flow and, hence, changed the distribution of the released GLP-1 between MLD lymph and HPV plasma in favor of MLD lymph. In addition, intestinal lymph contained less activity of DPP-IV, the major GLP-1 degrading enzyme, than HPV plasma. The resulting longer biological half-life of GLP-1 in intestinal lymph compared to HPV plasma, together with the changed distribution and the slower lymph flow compared to HPV blood flow presumably explains the higher GLP-1 concentrations and the longer duration of the increase in MLD lymph compared to HPV plasma. We also measured the MLD lymph and HPV plasma concentrations of GLP-1 after IP injection of the hormone at a dose that in our hands reliably inhibited short-term eating in rats under various conditions [4,33]. The moderate increase in MLD lymph and HPV plasma GLP-1 concentration that we detected after IP injection of 10 nmol/kg GLP-1 suggests that the satiating effect of this GLP-1 dose after IP administration is physiologically relevant. More specifically, that IP injection of 10 nmol/kg GLP-1, a dose that was previously shown to inhibit food intake, produced transient increases in the GLP-1 concentration in MLD lymph and HPV plasma at 20 and 40 min that can still be considered within the physiological range, indicates that this treatment may recapitulate the satiating effect of endogenous GLP-1. We had expected to find higher levels of active GLP-1 after GLP-1 administration in MLD than in HPV. This was clearly not the case, which indicates that HPV blood is a similarly viable readout for post-IP injection GLP-1 concentrations as is MLD lymph. The finding that there is no significant difference between MLD lymph and HPV plasma in this situation is nevertheless relevant because it suggests that at least under the conditions tested HPV blood can replace MLD lymph as a good readout for the microenvironment of the vagal afferent nerve terminals in the intestinal wall. The findings also suggest that the fat content of the meal influences the dynamics of the GLP-1 release and distribution. The greater GLP-1 appearance in MLD lymph after the HFD test meal may be due to the stimulating effect of dietary fat on intestinal lymph flow.

The question remains why the GLP-1 levels in HPV and MLD were rather low after IP injection of GLP-1. As we know from spiking experiments that the assay that we used detects the injected GLP-1, we can only speculate about the reasons for this. Perhaps a significant part of the IP injected GLP-1 was degraded by DPP-IV in the abdominal cavity or in any case before it reached our sampling sites. DPP-IV is a membrane bound en-

zyme expressed on the cell surfaces of most cells [1]. As an intrinsic membrane glycoprotein and a serine exopeptidase cleaving X-proline dipeptides from the N-terminus of polypeptides, DPP-IV is an enzyme that can cleave a large range of substrates. One of them is GLP-1 [10]. To address the question of possible changes in DPP-IV activity in response to a test meal and to compare its activity levels between MLD lymph and HPV plasma, we performed a DPP-IV activity assay. As expected based on the available literature [16,38], our measurements revealed a higher baseline DPP-IV activity in HPV plasma compared to MLD lymph, and neither the HFD nor the LFD test meal affected the DPP-IV levels. Interestingly, IP injection of 10 nmol/kg GLP-1 increased DPP-IV activity in HPV plasma, but not in MLD lymph. The GLP-1 (10 nmol/kg IP) induced increase in DPP-IV activity in HPV plasma was similar after both the HFD and LFD test meals and was presumably triggered by the increase in the HPV GLP-1 concentration. It is unclear why IP GLP-1 administration did not increase DPP-IV activity in MLD lymph despite the increase in MLD GLP-1 concentration. The low baseline activity and/or amount of DPP-IV in the MLD probably limits the potential of exogenous GLP-1 to increase the activity. As suggested earlier, the different flow rate [MLD lymph in a fasting rat 2-3 ml/h [16] < HPV blood in a sleeping rat approximately 20 ml/min [22]] together with the different DPP-IV activity (MLD < HPV) presumably contributed to the higher concentration of GLP-1 in MLD lymph compared to HPV plasma. Together, this also indicates that at least during HFD meals the vagal afferent terminals are exposed longer and to higher concentrations of GLP-1 (and probably other gut peptides) than one might estimate from HPV plasma. Insulin is released from the pancreas through the pancreatic vein into the HPV blood, i.e., it can reach MLD lymph only indirectly, via the systemic circulation and, ultimately, via the mesenteric artery and then diffusing from the capillaries into the interstitial fluid and, hence, intestinal lymph. It is therefore not surprising that the changes in MLD insulin levels induced by the meals and produced by the GLP-1 administrations mirrored the changes in HPV insulin levels, but at a much lower level. This is also true for MLD glucose concentrations, which showed a similar increase as HPV glucose after the LFD test meal, just starting from a lower level. Because insulin and glucose show similar postprandial profiles in MLD lymph and HPV plasma, measuring MLD lymph concentrations does not offer any advantages over measuring HPV plasma concentrations of these parameters.

As expected, TG were present at much higher levels in MLD lymph than in HPV plasma, and the HFD test meal resulted in higher TG levels in MLD lymph than the LFD test meal. Dietary fat-derived TG with long-chain fatty acids, which accounted for approximately 34.6% in our HFD test meal, are reesterified in the enterocytes, packed into chylomicrons, and transported away from the intestine via the lymphatic route, whereas medium-chain fatty acids released after digestion of TG molecules are absorbed via the HPV [16]. Interestingly, the high dose of IP GLP-1 dampened the rise in TG levels in MLD lymph after the HFD test meal. The most plausible explanation for this observation is the inhibition of gastric emptying that presumably occurred after the injection of GLP-1 and that slowed down the absorption and, hence, appearance of TG in MLD lymph. Previous studies addressing intestinal lymph composition were done using Bollman cages, in which the lymph is drained terminally from a severely restrained animal exposed to massive stress because no anesthesia is used [5]. In other studies in which Bollman cages were not used, animals were anesthetized and lymph was also drained terminally. The disadvantage of these procedures is that on one hand the anesthesia severely slows down lymph flow from 2-3 ml/h (reference) to 0.1-0.6 ml/h, thus also reducing the real time resolution in addition to any other effects that anesthesia may have on lymph composition [3, 16, 36]. In some studies a lymph fistula rat model without anesthesia was used for lymph collection, but the animals were still placed in restraint cages and, hence, subjected to a high level of stress that presumably affects the lymph production profile as well as the secretion of gut peptides [16, 20, 40]. This could also affect the GLP-1 measurements because stress was previously linked to the cleavage of DPP-IV from the cell surfaces into the blood stream, hence changing the physiologically relevant concentrations of active GLP-1 [18, 29, 30].

Our model avoids the problems related to anesthesia and stress by implanting MLD, HPV and IP catheters. Allowing the animals to fully recover from the surgery and regain their pre-surgical BW and handling them daily prior to sampling, minimizes the stress level during our experiments. Rats are recovering for at least one week post-surgery prior to experiments performed on them. Also, no restraint is needed during the sampling, and in general, the animals can freely move and eat the test meal while corresponding drug injections are done or blood and lymph samplings are performed. As mentioned above, our method allowed for the first time to sample HPV blood and MLD lymph in parallel, thus permitting for a di-

rect comparison between the secretome of these two fluid compartments. Moreover, because our method allows for multiple sampling time points it is very useful to monitor both the secretion and degradation profiles and thus gives a better idea about the life-time of different measurable compounds in lymph compared to the blood.

Perspectives and significance: Our findings support IP administration as an adequate route of administration for investigations into the physiological relevance of GI peptides. In addition, the present study as well as previous experiments [2] document the usefulness of MLD lymph sampling for investigations into the in-vivo GI peptide release and function in relation to eating, in particular when fat rich food is consumed. This is because then intestinal lymph provides a better readout of the interstitial fluid of the intestinal wall than HPV blood. Employing intestinal lymph samplings and measurements in relation to studying in-vivo enteroendocrine cell function with and without involvement of the extrinsic innervation of the intestine can produce very interesting results. To realize the full potential of this novel technique, however, it should be employed addressing phenomena that can not, or can at least only hardly, be detected in HPV blood or elsewhere in the systemic circulation. Thus, intestinal lymph sampling, perhaps together with tracing techniques, might be useful to examine the release of lipids that are temporarily stored in the wall of the small intestine after a fat containing meal [12, 21, 28, 41]. This is a well-documented, but poorly understood phenomenon. Other potential applications relate to immune functions of the intestinal wall, perhaps in conjunction with modifications of the gut microbiome, a currently very fashionable topic of research [14, 24, 37]. In addition, lymph and blood parallel sampling technique could be applicable for a better understanding of the antiobesity and antidiabetic effects of bariatric surgery. Because previous studies have shown the effective upregulation of some GI peptides after bariatric surgery [26], further investigations of their compartmentalization and functional relevance would be of a great importance. Overall, we hope that the unrestrained rat intestinal lymph sampling model, in combination with other in vivo and in vitro analyses, will provide further useful data and help to better understand gut biology as well as gut-brain interactions.

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4

GENERAL DISCUSSION

4.1 OVERVIEW OF THE MAIN FINDINGS

The results presented in the two manuscripts that are included in this thesis contribute on one hand to a better understanding of the role of PPG neuron regulation (possibly central GLP-1 regulation). On the other hand, they provide a critical test of an animal model that supposedly allows for the precise monitoring of the release of peripheral GLP-1 in vivo and, hence, for better studying its function.

In the study described in Chapter 2 of the thesis, our aim was to expand current knowledge and gain more insight into the mechanism of how PPG neurons in the NTS contribute to the regulation of energy homeostasis. We specifically investigated the role of PPG neurons, the main producers of central GLP-1, in mediating the control of energy intake and expenditure. Interestingly, PPG mRNA levels were upregulated after HFD feeding and in all animal models with altered leptin signalling that we tested. This suggests leptin as a candidate PPG regulator. We also showed (quantified) for the first time that HFD exposure actually increased the number of PPG expressing neurons in the NTS, raising the question, which are those additional neurons in the NTS that start PPG production and presumably contribute to the overall PPG mRNA increase? With the aim of better understanding the PPG mRNA upregulation and its physiological relevance, we performed a bilateral PPG KD in WT mice, and demonstrated that PPG KD decreased FI and BW gain. In addition we addressed consequences of PPG neuronal modulation, using DREADD virus in Glu-Cre DIO animals. Our results indicate that a chronic increase in central PPG levels (possibly GLP-1) with HFD feeding contributes to the development of obesity by modulating neural circuits involved in the control of EI and EE. In turn, acute changes in PPG neuronal activity presumably recruit separate CNS circuits to produce opposite effects on FI (activation a decrease and silencing an increase, respectively) compared to the PPG KD.

In the studies described in Chapter 3 of the thesis, we focused on peripheral rather than central GLP-1 and wanted to examine MLD lymph

as a potentially better readout than HPV plasma for monitoring intestinal GLP-1 secretion. We used and refined a previously developed tool to precisely investigate the release of peripheral GLP-1. We sampled MLD lymph and HPV blood over time, in relation to the consumption of isocaloric test meals with different fat contents, with the aim of precisely measuring the endogenous GLP-1 release, as well as addressing the possible physiological relevance of IP administered GLP-1. Our findings indicate that the dynamics of GLP-1 appearance in MLD lymph and HPV plasma are different, due to various factors including differences in DPP-IV activity in these two compartments and the fat content of the meal. Overall, however, MLD lymph did not provide a better readout for endogenously released GLP-1 than HPV plasma. Nevertheless, intestinal lymph may have advantages for other applications, such as investigations into postprandial fat metabolism or intestinal immune functions.

4.2 CENTRALLY AND PERIPHERALLY PRODUCED GLP-1

To address the role of central GLP-1, it is important to differentiate its function from the function of peripherally produced GLP-1, or to consider how and where these two possibly interact. This raises several critical questions: 1) Does only centrally produced GLP-1 have the capacity to reach the GLP-1 receptors in the brain because it is released locally? 2) Or does some peripherally derived GLP-1 also gain direct access to GLP-1 receptors within the CNS despite its short half-life? 3) Or does peripheral GLP-1 signal the CNS only through afferent nerve connections?

4.2.1 *Do peripheral and central GLP-1 have different roles?*

The following effects, which were reported by several laboratories, generate a dilemma:

1. Plasma levels of GLP-1 are downregulated in DIO [1–3].
2. PPG mRNA is upregulated in DIO in mice [4] (and our own data).
3. PPG mRNA is upregulated in situations when leptin signalling is altered in mice [4] (and our own data).

Thus, do peripheral and central GLP-1 have different functions, and why do peripheral and central GLP-1 appear to change in opposite directions? Of course, one needs to consider that we talk here about an 'increase' in

central GLP-1, but what was reported is not an increase in GLP-1 itself but rather in the mRNA level of its precursor PPG, which does not necessarily mean an increase in the GLP-1 level. PPG gives rise to several different peptides [5], and it is conceivable that the posttranscriptional processing of the PPG changes in different situations.

Peripheral administration of long-lasting GLP-1R agonists has shown beneficial effects on BW and glycaemic control [6–10]. GLP-1R agonists are therefore a viable treatment option for obese and diabetic patients [1,11,12]. As these compounds appear to act mainly centrally [5,9], it appears counterintuitive that GLP-1, a known satiating signal, should be upregulated in the brain of DIO mice [4], while its level has been shown to be lowered in the periphery [3]. Moreover, according to our data and other findings, downregulation of central PPG production and blocking of central nervous system GLP-1R signalling have unexpected effects on body weight (BW) and glycaemic control [13–15]. ICV injection of the GLP-1R antagonist exendin-9 (Ex9) in DIO mice in fact improved these animals' metabolic phenotype [15]. This finding highlights the possibility of negative effects of excess central GLP-1R signalling and suggests that DIO is a state of central GLP-1 resistance. It would be worth testing whether central GLP-1 overexpression is a cause or consequence of the DIO phenotype.

This raises an interesting question: Is it possible that a reduction in peripheral GLP-1 may cause an increase in central GLP-1 production? In other words, is there a negative feedback mechanism at work between the peripheral and central GLP-1 systems? PPG neurons themselves do not express GLP-1 receptors, excluding the possibility that peripheral GLP-1 directly affects central PPG neurons to inhibit GLP-1 production. Hence, some other signals than GLP-1 presumably modulate central PPG upregulation and GLP-1 release. This, of course, does not exclude the possibility that these other signals might be directly or indirectly controlled by peripheral GLP-1.

4.2.2 *Possible mechanism of PPG upregulation*

In the experiments presented in Chapter 2, we showed that mice kept on regular CD express functional leptin receptors on PPG neurons. Previous studies have shown that leptin depolarizes PPG neurons derived from chow-fed mice, which demonstrates that these neurons are leptin-

sensitive [16]. It is however unknown whether PPG neurons remain leptin-sensitive in DIO. We attempted to address this question using HFD-fed GLU-YFP mice to visualize PSTAT-3 activation in PPG neurons of DIO mice, but we failed to reliably demonstrate PSTAT-3 staining, making it impossible to draw conclusions about the leptin sensitivity of these neurons. When we performed the PSTAT-3 staining, to verify the staining itself we had included 10 week-old mice as a positive control, and confirmed that the staining worked. In our HFD experiments, however, there were no CD animals as control. Therefore, after there was no PSTAT-3 positive staining after either leptin or saline injection in the HFD animals, the lack of an appropriate control unfortunately did not allow us to draw any conclusion with respect to leptin sensitivity or resistance of these neurons. Another interesting experiment to address the question of whether PPG neurons are leptin resistant after chronic HFD exposure would be to perform electrophysiological recordings from PPG neurons of HFD fed obese animals and examine whether the addition of leptin would still lead to depolarization of these neurons.

Our experiments indicated that even though leptin can regulate PPG neurons, it is not a major driver of central PPG mRNA upregulation. Other molecules should be involved. Because DIO is associated with chronic inflammation and an increase in secretory pro-inflammatory cytokines [17,18], it could be that these cytokines – or some of them – upregulate central PPG expression. One viable candidate may be the pro-inflammatory cytokine interleukin-6 (IL-6). Data showing that IL-6 stimulates peripheral GLP-1 secretion from the intestine and pancreas and that PPG neurons have functional IL-6 receptors (IL-6R) support this idea [19]. On one hand it was shown that GLP-1 reduces inflammatory markers [20–25], on the other hand GLP-1 has the capacity to enhance inflammatory signalling [26]. Interestingly, IL-6 appears to be involved in the central mediation of the FI and BW effects of GLP-1 in rats and mice [27] because blockade of central IL-6 (and IL-1) signalling eliminated these effects of Ex4. Other studies suggest that leptin increases brain interleukin signalling [28, 29]. The CNS cell types producing IL-6 in response to peripheral signals remain unclear, but various studies point to neurons, astrocytes, and microglia as potential GLP-1-interleukin interaction targets. Interestingly, microglia in an activated state can also produce GLP-1 [22,30], but the relevance and possible function of this phenomenon has scarcely been examined. Considering the protective properties of GLP-1 in the brain, it is possible that elevated cen-

tral GLP-1 provides an additional neuroprotective function [31] during the state of chronic low inflammation such as DIO.

All in all, there seem to be bidirectional interactions between IL-6 and GLP-1, and perhaps other cytokines as well, and pathophysiological states such as obesity might further enhance the relevance of these interactions. Importantly, the relationship of central GLP-1 with inflammatory signals has not yet been extensively studied in the context of the chronic inflammatory state of obesity. To do so, one could do in vivo screenings with IP injections of pro-inflammatory cytokines at different concentrations and examine if this would lead to an upregulation of central PPG mRNA.

4.2.3 *Can PPG KD prevent DIO?*

In order to address the potential of central PPG downregulation in the prevention of obesity, a useful experiment would have been to KD PPG using a LV-shPPG before the development of obesity. That is, perform the KD in mice on CD, then switch them to a HFD and test whether those mice might be protected from the DIO phenotype or at least be spared some of its aspects compared to LV-control infected animals. Unpublished data (not included in this thesis) from an experiment in which we performed the LV-mediated PPG KD in animals fed CD suggest that PPG KD might in fact have a protective effect because these mice gained less weight over time compared to LV-control animals and showed overall the same outcomes when compared to the HFD KD experiments. However, in those experiments we failed to confirm the KD in animals fed CD and, hence, could not use the data.

In fact, all experiments with PPG KD animals on HFD were also done in parallel in animals kept on CD, and the results tended to be similar, hence supporting our major findings and interpretations. Unfortunately, however, the LV-induced KD of PPG in chow-fed animals turned out to be not significant; therefore, I did not include the results in this thesis. The surgeries were performed exactly at the same time, the animals were from the same breeders, and the virus used was from the same badge. Therefore, we can only speculate about the reasons for the difference between HFD and CD-fed animals. One possibility is that the KD was perhaps more efficient when PPG was upregulated, as in HFD-fed DIO mice, than when

it was not increased as in CD-fed animals. Further experiments should address this possibility.

In addition, we found that the PPG KD animals had decreased FI and BW compared to the control animals. We did not perform any behavioural test in these animals to examine whether they were eating less because of enhanced satiation and satiety or of other behavioural consequences (e.g. feeling sick). Without this crucial piece of information, it is difficult to speculate about a possible mechanism of the effect of the PPG KD on eating.

Interestingly homozygous glucagon-GFP knock-in mice lacking PPG derived-peptides including GLP-1 were shown to display glucose intolerance similar to their heterozygous controls after 15-20 weeks of HFD exposure, but had lower BW gain and exhibited increased oxygen consumption. Overall these findings suggest that the absence of proglucagon-derived peptides results in resistance to the obesity phenotype [32]. This raises the possibility that central GLP-1 signalling outweighs the, or dominates over, peripheral GLP-1 signalling, or that peripheral and central GLP-1 have different functions.

To further advance our understanding of the role of specifically central GLP-1, it would have been similarly interesting to generate mice with a global PPG KD and re-express PPG only centrally. This should also help to dissect the functions of peripheral and central GLP-1. The down-side of this approach is, however, that when mice are genetically modified to lack a certain gene from birth, other genes often compensate for the absence of the gene of interest, i.e., there may be no particular phenotype. One vivid example are the GLP-1R KD mice with a targeted disruption of the GLP-1R (confirmed with ICV administration of GLP-1, which inhibited FI in WT but not in GLP-1R KO mice). These mice exhibit a rather 'mild' metabolic phenotype with diminished levels of circulating insulin, but no evidence for abnormal BW or FI behaviour [33].

4.2.4 *Species differences*

All experiments of this thesis on the role of central PPG were done in mice. Therefore, we cannot exclude that some of the phenomena that we saw, might differ among species. This might also apply to our experiments

with PPG mRNA upregulation after HFD feeding, when leptin signalling was altered. Some of the discrepant findings in the literature may also be related to species differences. To further emphasize this point I would like to mention that there is a striking difference between mouse and rat PPG neurons. Mouse PPG neurons have leptin receptors and are clearly regulated by leptin, i.e., leptin depolarizes PPG neurons from mice kept on a regular CD [16]. Rat PPG neurons on the other hand are not regulated by leptin [4]. In addition, fasting mice results in lower central PPG mRNA levels, and leptin treatment re-establishes pre-fasting levels. In contrast, neither fasting nor leptin treatment alters central PPG mRNA levels in rats [4]. These species differences raise the question of whether this phenomenon (the PPG overexpression in DIO models) only occurs in mice or whether it is also relevant for human obesity. To my knowledge there are no published data in humans addressing central levels of PPG in obesity, hence the relevance of PPG expression in humans has yet to be established.

4.3 FINAL REMARKS

With the work presented in this thesis, we aimed at contributing to a better understanding of the role of centrally produced GLP-1 in BW homeostasis. We employed several experimental models to address 1) the role of leptin in the regulation of PPG expression by blocking leptin signalling in DIO mice, 2) the role of HFD-induced PPG overexpression in the regulation of BW and food intake using viral-mediated RNA interference, and 3) a different way of PPG neuronal manipulation by using the DREADD technology. In showing that PPG neurons contribute to both aspects of energy homeostasis, that is EI and EE, the obtained results expand current knowledge about the role of central PPG in the regulation of energy balance and raise additional important questions, which need to be addressed in the future.

We also addressed the question of whether MLD Lymph could be used as a readout for peripheral GLP-1 and metabolite release. In our particular experimental setup, the GLP-1 measurements in intestinal lymph did not provide additional insights. Nevertheless, this model could still be useful for addressing different questions, for instance to examine whether the sampling of intestinal lymph could be a valuable tool to monitor the release of other intestinal peptide or metabolites derived from enterocytes, or for questions in relation to fat absorption and intestinal immunity. Over-

all, the results raise several questions and suggest future experiments that could not be performed anymore within the scope of this thesis. I believe, however, that if further pursued, the proposed approaches and the animal models developed would allow for further improvements of our basic understanding of the diverse roles of central and peripheral GLP-1 in regulating energy homeostasis.

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APPENDIX

Curriculum Vitae

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PERSONAL

31 years old, Georgian
Swiss permit B

EDUCATION

PhD student, in Life Sciences
Swiss Federal Institutes of Technology (ETHZ), Zürich, Switzerland
Expected in February 2018
Specialization: Neuroscience

Master of Science, in Life Sciences
Swiss Federal Institutes of Technology (EPFL), Lausanne, Switzerland
February 2011
Specialization: Bioengineering and Biotechnology

Bachelor of Science, in Physics
Tbilisi State University (TSU), Tbilisi, Georgia
August 2007
Specialization: General Physics

TECHNICAL SKILLS

Chemistry: Photolysis of compounds, Solid phase peptide synthesis, Native chemical ligation.
Biochemistry: Expression of proteins in E.coli, Purification of proteins (using: anion and cation exchange chromatography, hydrophobic/hydrophilic interaction chromatography, reverse-phased (RP-HPLC) chromatography) Western Blot analysis, Native page, Dot blot, MALDI and ESI analysis.
Molecular biology: Single-point mutagenesis, Classical cloning, PCR, RNA extraction quantification.
Biophysics: Protein oligomerization assays, Circular dichroism, Thioavin T fluorescence assay.
Cellular biology: Cell culture, Transfections, Isolation of embryonic primary cortical/hippocampal neurons, Cytotoxicity assays, Immunocytochemistry, Microvescile and Exosome isolation.
Microscopy: Transmission electron microscopy, Wide-field fluorescence microscopy, Confocal microscopy.
Animal experimentation: Handling mice and rats, blood sampling, dissections, perfusion.
Animal behavior: Plus maze, open field, fear conditioning, Y-maze, social interaction.
Surgery: Stereotaxic injections, 3rd and 4th ventricular cannulation in mice, implantation of hepatic portal vein, jugular vein and vena cava cathetars in rats.

RESEARCH EXPERIENCE

Phd Student 2012 - Now
Laboratory of Physiology and Behavior in the Institute of Food, Nutrition and Health
ETH Zürich, Switzerland

- Investigating on leptin regulation of preproglucagon neurons.
- Influence of GLP-1 on mice behavior in high fat diet conditions.
- Lymph and blood parallel sampling in unrestrained rat model.

Laboratory experience 2011 - 2012
Laboratory of Systems and Cell Biology of Neurodegeneration in the Division of Psychiatry Research, UZH, Zurich, Switzerland 2011 - 2012

- Working on cellular basis of the release and transmissibility of amyloidogenic proteins.
- Investigating the origin of presence of cytosolic proteins in the human cerebrospinal fluid (CSF).
- Performing characterization of Amyloid beta aggregates, isolation of different aggregated species and their further use on cytotoxicity studies.

Master thesis project 2010 - 2011
Laboratory of Molecular Neurobiology and Functional Neuroproteomics (LMNN), EPFL, Lausanne, Switzerland

- Working on characterization and applications of a novel chemical tool with photolable linker to control protein/peptide folding and amyloid formation.

Engineer 2006 - 2007
Georgian National Found, Tbilisi, Georgia

- My responsibilities included conducting various calculations using Numerical methods like FDTD and MAS.

Researcher 2003 - 2007
Laboratory of Applied Electrodynamics, TSU, Tbilisi, Georgia

- Involved in a project concerned with investigation of "The influence of electromagnetic radiation on living objects", where we developed software to simulate different physical phenomena, especially from electromagnetic field theory.

COMPUTER SKILLS

Languages & Software: Fortran, Delphi, Pascal, Python, Java, Mathematica, LaTex, Eclipse, Netbeans, Web development.

OPEN INITIATIVES

2016 - Developed recycling event converter for Schwerzenbach Gemeinde

- Python-based event converter for calendar integration
- Supports Google and MS Outlook calenders

2015 - Co-Organizer of a joint EPFL - ETHZ summer school in Translational Biology

- Event initiator and coordinator from ETHZ side
- Recruitment and management of volunteers for organizing the event
- Management of administrative aspects
- Speakers selection and invitation
- Marketing activities for popularizing the event

2010 - Extending features and capabilities of the open source digital library system Invenio, maintained at CERN by CERN Document Server (CDS) group.

- Suggested usability improvements
- Extended language pack to support new languages

PUBLICATIONS

1. **Jejelava N.**, Langhans W., Shin J. L., "Pre-Pro-Glucagons neuronsin in regulating energy homeostasis ." **In preparation**.
2. **Jejelava N.**, Kaufman S., Krieger JP., Terra MM., Langhans W., Arnold M., "Intestinal lymph as a readout of meal-induced GLP-1 release in an unrestrained rat model."Am J Physiol Regul Integr Comp Physiol. 2018 Jan 17**.**
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10. Zaridze R., Kakulia D., Kajaia G., Mazmanov D., Manukyan L., **Jejelava N**., Gogua T. "EM Field Propagation Outdoors and Indoors and Unconfined Spaces". Proceedings of the European Conference on Antennas & Propagation (EuCAP 2006), November 6-10, 2006, Nice, France.

POSTER PRESENTATION

- **Jejelava N.**, Langhans W., Lee J. S., *Increased central glucagon like peptide-1 (GLP-1) production contributes to high fat diet (HFD)-induced obesity (DIO) SSIB 2016, the 24th Annual Meeting of the Society for the Study of Ingestive Behavior,* Portugal, Porto July 2016
- **Jejelava N.**, Thürlemann M., Langhans W., Lee J. S., *Leptin receptor in GLP-1 neurons contributes to diet-induced obesity Innovation for Health,* Netherlands, Rotterdam February 2016

- **Jejelava N.**, Arnold M., Langhans W., *Characterization of Glucagon-like peptide-1 (GLP-1) release and distribution in blood and lymph using the unrestrained rat model,* Switzerland, Ascona, September 2014
- Minakaki G., **Jejelava N.**, Surendranath V., Rajendran L., *On the origin of cytosolic proteins in the cerebrospinal fluid,* Tag der Forschung der Psychiatrie und Kinderund Jugendpsychiatrie, Switzerland, Zurich, December 2011
- Awad L., **Jejelava N.**, Brik A., Lashuel H., *Novel chemical tools to facilitate the synthesis and control the folding and Self-assembly of amyloid-forming polypeptides,* The 22nd American Peptide Symposium, USA, San Diego, June 2011
- Awad L., **Jejelava N.**, Brik A., Lashuel H., *Novel chemical tool to control folding and amyloid-formation,* The EPFL-ETH-Tokyo Tech Joint Symposium, Switzerland, Lausanne, June 2011

CERTIFICATIONS AND AWARDS

- 2011 - LTK module 1.
- 2008 - Development and research scholarship for MS studies.
- 2007 - Excellence scholarship in BS studies.
- 2006 - First Degree Diploma in programming and computer modelling, at 66^{th} student's conference (Georgia, Tbilisi).
- 2005 - Second Degree Diploma in programming and computer modelling, at 65^{th} student's conference (Georgia, Tbilisi).
- 2005 - Diploma in physics at 65^{th} student's conference (Georgia, Tbilisi).
- 2004 - Third Degree Diploma in physics at 64^{th} student's conference (Georgia, Tbilisi).
- 2003 - Diploma in actual of physics at student's conference (Georgia, Tbilisi).
- 2003 - Certificate in 16^{th} International Young Physicists' Tournament (Sweden, Uppsala).
- 2003 - Second Degree Diploma at 12^{th} Republic Young Physicists' Tournament (Georgia, Tbilisi).
- 2003 - First Degree Diploma at South Caucasus 3^{rd} Regional Young Environmentalists' Tournament (Georgia, Tbilisi).
- 2002 - Third Degree Diploma in biology, at Republic Olympiad (Georgia, Tbilisi).
- 2001 - Third Degree Diploma in biology, at Republic Olympiad (Georgia, Tbilisi).
- 2000 - Third Degree Diploma in biology, at Republic Olympiad (Georgia, Tbilisi).
- 1999 - Won award in biology, at City Olympiad (Georgia, Tbilisi).

LANGUAGES

Georgian	Russian	English	German
Mother tongue	Fluent	Fluent	Intermediate

EXTRA-CURRICULAR ACTIVITIES

- 2015-now - Member of Schwerzenbach Tennis club.
- 2014-now - Founder of startup for producing educational toys and puzzles.
- 2004-2007 - Member of the Tbilisi State University girl's basketball team.
- 1995-2002 - School of music with specialization in Fortepiano.